The Role of Human MutL Proteins in DNA Repair

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1. Summary

Mismatch repair (MMR) is primarily involved in the correction of replication errors. As MMR reduces the mutation frequency up to three orders of magnitude, it makes a major contribution towards accurate transmission of genetic information. Mammalian MMR proteins are conserved through evolution. In human cells, recognition is mediated by two homologues of the *E.coli* MutS protein, hMutSα, a heterodimer of hMSH2 and hMSH6, or hMutSβ, a heterodimer of hMSH2 and hMSH3. Subsequently hMutLα, a heterodimer of hMLH1 and hPMS2, both homologues of the bacterial MutL protein, is thought to couple mismatch recognition with downstream events that include strand discrimination, exonucleolytic degradation of the mismatch-containing strand (by an exonuclease such as EXO1) and final resynthesis mediated by the DNA polymerase δ. Defects in MMR lead to increased mutagenesis, and were linked to colorectal cancer. Hereditary Non-Polyposis Colon Cancer (HNPCC) occurs with a very high frequency in individuals carrying germline mutations in one allele of primarily *hMLH1* or *hMSH2*. In addition, epigenetic inactivation of *hMLH1* through promoter hypermethylation can result in sporadic cancer. Together, these mechanisms account for about 15% of colorectal cancer cases worldwide. In addition, cells lacking MMR are resistant to several types of DNA damaging agents, including those used in cancer chemotherapy, such as the methylating drug temozolomide and the crosslinking agent cisplatin.

Although the role of hMutSα and hMutSβ in mismatch recognition and binding has been extensively described, the exact function of the MutL proteins remains mostly unclear. I set out to gain a better understanding of the function of these polypeptides in MMR and/or related processes. In human cells, hMLH1 can bind to three MutL homologues, hPMS2,

hPMS1 or hMLH3, to form the heterodimers hMutLα, hMutLβ and hMutLγ respectively (Fig.1).

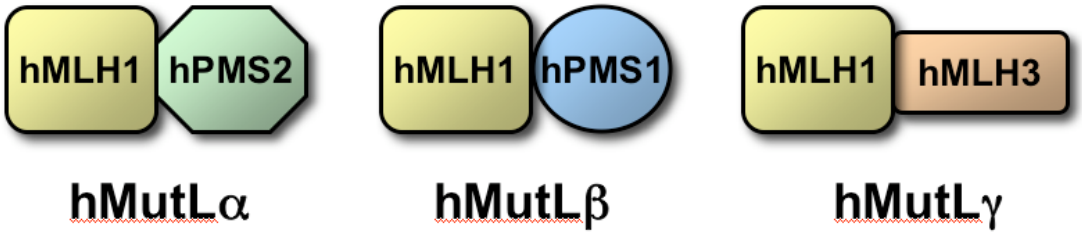

Fig.1 MutL heterodimers in human cells.

Of the three human heterodimers, only hMutLα is absolutely required for MMR. The role of hMutLβ is still unknown; although it was shown to bind hMutSα, it lacks any MMR activity *in vitro*. Similarly, the function of hMutLγ in MMR remained unknown, although it participates in meiotic recombination.

The first part of my studies was aimed at the characterization of the function of the hMutLγ heterodimer in human cells with respect to its possible role in MMR. The results obtained represent the first report on the expression of hMLH3 in human cells and its possible role in MMR. We could show that hMLH3 is present at very low amounts in human cell extracts and that it physically interacts with hMLH1. The presence of hMLH1 is essential for the stability of hMLH3 in Sf9 cells. However, this interaction is not necessary for the stabilization of hMLH3 in human cells, since we found several hMLH1-deficient cell lines expressing hMLH3. In this respect hMLH3 differs from the other major hMLH1-interacting partners, hPMS2 and hPMS1, which are highly unstable in the absence of hMLH1. Our finding suggested that hMLH3 might have an alternative interacting partner that can mediate its stability. Interestingly, hMLH3 expression can be silenced by promoter hypermethylation. To test the role of hMutLγ in MMR, we used cell extracts from Sf9 cells expressing hMLH3 to complement the repair defect of extracts prepared from hMutLα-

deficient cells. Our *in vitro* MMR assays showed that hMLH3 can play a minor role in MMR, acting together with hMutSα in the repair of single mismatches and small insertion-deletion loops. Nevertheless, hPMS2-deficient cells expressing hMLH3 still show a strong MMR-defect, suggesting that a major role of hMLH3 at physiological concentrations *in vivo* is unlikely. However, even a marginal role of hMLH3 in MMR, in partial redundancy with hPMS2, and its different expression levels *in vivo*, might help to explain the low penetrance of *hPMS2*-mutations in HNPCC, compared to *hMLH1*-mutations.

MMR is critically dependent on the assembly of multimeric complexes involved in mismatch recognition and signal transduction to downstream repair events. Although the human MMR was reconstituted *in vitro* and thus the minimal protein requirements are known, the mechanism taking place *in vivo* is still not fully understood, especially in terms of regulation, redundancy and the link to other pathways such as recombination or activation of apoptosis. Moreover, the exact biological function of the heterodimer hMutLα (hMLH1 and hPMS2) is still enigmatic. The second part of my graduate studies was focused on the analysis of proteins interacting with hMutLα. We believe that the identification of novel interacting partners of hMLH1 and hPMS2 could yield essential information about the role of these two proteins in MMR and/or in other DNA repair-related mechanisms. To this aim we used Tandem Affinity Purification (TAP), a recently developed two-step technique that allows the purification of interactors under native conditions and has been shown to be superior to one-step pulldown methods in terms of accuracy and specificity. We stably transfected two cell lines deficient for hMLH1 or hPMS2, namely 293T and HeLa12, with a vector expressing the N-terminally TAP-tagged hMLH1 or C-terminally TAP-tagged hPMS2, respectively. We could show that the tagged constructs corrected the MMR-defect of the two respective cell lines both *in vitro* and *in vivo*. Whole cell extracts from these cell lines were used for TAP, the eluates were loaded

on a polyacrylamide gel and the bands were cut out and analyzed by Mass Spectrometry (MS). We validated the technique by the detection of the bait proteins and their major known interacting partners. We could identify a number of proteins specifically present in the respective TAP eluate. Some of these interactors had already been described, but the majority of them represented novel findings. Most notably, the recently identified FancJ factor BACH1 (or BRIP1), was specifically present at high amounts in our pulldowns. We could confirm this interaction by independent techniques and currently are actively investigating its role in MMR. Moreover, since there are no hPMS1-deficient cell lines to allow for the complementation of the defect by TAP-tagged hPMS1 construct, we performed a large-scale immunoprecipitation coupled to MS analysis to identify the interacting partners of hPMS1. Interestingly, among the proteins that we identified we found a number of factors belonging to the ubiquitin pathway. In summary, using the TAP technique and the large-scale immunoprecipitation, we identified numerous polypeptides that are specifically present in complexes with hMLH1, hPMS2 and hPMS1 that we listed according to their known biological functions. We started to validate the most interesting interactions and characterize their biological roles in the hope to better understand the MMR process *in vivo*.

2. Zusammenfassung

Die Fehlpaarungs-Reparatur (Mismatch Repair, MMR) ist hauptsächlich für die Korrektur von Replikationsfehlern verantwortlich. Da sie die Mutationsrate um das Tausendfache reduziert, spielt dieses Reparatursystem eine Hauptrolle in der fehlerfreien Übertragung von genetischer Information. Die MMR-Proteine von Säugetieren sind hochkonserviert. In menschlichen Zellen wird die Erkennung einer Basen-Fehlpaarung durch zwei Homologe des *E. coli* MutS-Proteins, hMutSα (ein Heterodimer aus hMSH2 und hMSH6) oder hMutSβ (ein Heterodimer aus hMSH2 und hMSH3) gewährleistet. hMutLα, ein Heterodimer bestehend aus den zwei bakteriellen MutL-Proteinhomologen hMLH1 und hPMS2, verknüpft dann den Fehlpaarungs-Erkennungsschritt mit nachfolgenden Ereignissen, zu welchen Strang-Diskriminierung, exonukleolytische Degradierung des fehlgepaarten Stranges (durch eine Exonuklease wie zum Beispiel EXO1) und die Resynthese durch DNA-Polymerase δ gehören. Defekte in der MMR führen zu erhöhter Mutagenese und nachweislich zu kolorektalem Krebs. Träger von Keimbahnmutationen in einem Allel des *hMLH1*- oder *hMSH2*-Gens erkranken mit sehr grosser Wahrscheinlichkeit an hereditärem nicht-polypösem Dickdarmkrebs (Hereditary Non-Polyposis Colon Cancer, HNPCC). Zusätzlich kann *hMLH1* durch Promotor-Hypermethylierung epigenetisch inaktiviert werden und als Folge davon vereinzelt (sporadisch) Krebs entstehen. Zusammen machen diese Mechanismen etwa 15% der weltweit auftretenden kolorektalen Krebsfälle aus. Nennenswert ist zudem, dass Zellen ohne MMR Resistenzen gegenüber verschiedenen Arten von DNA-schädigenden Wirkstoffen entwickeln, auch gegenüber solchen, welche in der Krebs-Chemotherapie eingesetzt werden, wie zum Beispiel Temozolomid (methylierender Wirkstoff) und Cisplatin (quervernetzendes Agens).

Obgleich die Rolle von hMutSα und hMutSβ im Prozess der Erkennung und Bindung einer Basen-Fehlpaarung ausführlich beschrieben ist, bleibt die genaue Funktion des MutL-Proteins mehrheitlich unklar. Mein Ziel war es, ein besseres Verständnis über die Funktion dieser Polypeptide in der MMR und/oder verwandten Prozessen zu gewinnen. In menschlichen Zellen kann hMLH1 an die drei MutL-Homologe hPMS2, hPMS1 oder hMLH3 binden und bildet so die Heterodimere hMutLα (hMLH1-hPMS2), hMutLβ (hMLH1-hPMS1) oder MutLγ (hMLH1-hMLH3) (Fig. 1).

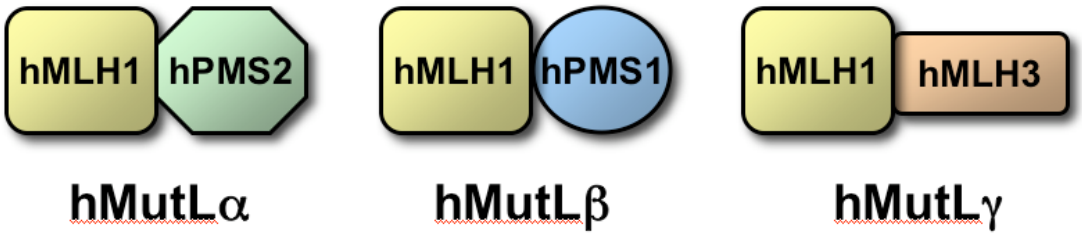

Fig.1 MutL-Heterodimere in menschlichen Zellen

Von den drei menschlichen Heterodimeren wird nur hMutLα zwingend für die MMR benötigt. Die Rolle von hMutLβ ist nach wie vor unbekannt; obschon gezeigt wurde, dass hMutLβ an hMutSα bindet, konnte keine MMR-Aktivität *in vitro* nachgewiesen werden. Unbekannt bleibt auch die Funktion von hMutLγ in der MMR, wobei man weiss, dass dieses Heterodimer an der meiotischen Rekombination beteiligt ist.

Der erste Teil meiner Doktorarbeit befasste sich mit der Charakterisierung der Funktion von hMutLγ in menschlichen Zellen und der Frage, welche mögliche Rolle dieses Heterodimer in der MMR spielen könnte. Meine Resultate demonstrierten zum ersten Mal die Expression von hMLH3 in menschlichen Zellen und dessen mögliche Rolle in der MMR. Wir konnten zeigen, dass hMLH3 in sehr geringen Mengen in menschlichen Zell-Extrakten vorhanden ist und physisch mit hMLH1 interagiert. Die Anwesenheit von

hMLH1 ist essenziell für die Stabilität von hMLH3 in Sf9-Zellen. In menschlichen Zellen ist diese Interaktion jedoch nicht notwendig für die Stabilisierung von hMLH3, da wir in einigen hMLH1-defizienten Zell-Linien die Expression von hMLH3 beobachten konnten. Demzufolge unterscheidet sich hMLH3 von den beiden anderen hMLH1-Hauptinteraktionspartnern hPMS2 und hPMS1, welche im höchsten Masse instabil sind in der Abwesenheit von hMLH1. Wir vermuten, dass hMLH3 möglicherweise alternative Interaktionspartner hat, welche die Stabilität des Proteins gewährleisten. Interessanterweise kann die hMLH3-Expression durch Promotor-Hypermethylierung ausgeschaltet werden. Um die Rolle von hMutLγ in der MMR zu testen verwendeten wir Zellextrakte von hMLH3-exprimierenden Sf9-Zellen und komplementierten damit den Reparaturdefekt von hMutLα-defizientem Extrakt. Unser *in vitro*-MMR-Ansatz zeigte, dass hMLH3 zusammen mit hMutSα eine untergeordnete Rolle bei der Reparatur von Einzel-Fehlpaarungen und kleinen Insertions-Deletionsschlaufen spielt. Nichtsdestotrotz zeigen hMLH3-exprimierende, hPMS2-defiziente Zellen immer noch einen starken MMR-Defekt, was darauf hindeutet, dass hMLH3 bei physiologischen Konzentrationen *in vivo* keine bedeutende Rolle zukommt. Jedoch kann nicht ausgeschlossen werden, dass hMLH3 in teilweiser Redundanz mit hPMS2 eine marginale Rolle in der MMR spielt und die unterschiedlichen hMLH3-Expressions-Levels *in vivo* erklären möglicherweise die tiefe Penetranz von *hPMS2*-Mutationen in HNPCC im Vergleich zu *hMLH1*-Mutationen.

Für die MMR ist die Assemblierung von multimeren Komplexen, welche in die Basen-Fehlpaarungs-Erkennung und Signal-Weiterleitung an nachgeordnete Reparaturprozesse involviert sind, von entscheidender Wichtigkeit. Obschon die menschliche MMR *in vitro* rekonstituiert wurde und die minimalen Protein-Anforderungen bekannt sind, ist der *in vivo*-Mechanismus nach wie vor nicht vollständig entschlüsselt. Insbesondere fehlen Informationen über die Regulation, die Redundanz und die Verbindung zu anderen Pfaden

wie Rekombination oder Apoptose-Aktivierung. Zudem bleibt die genaue biologische Funktion des Heterodimers hMutLα ein Mysterium.

Der zweite Teil meiner Doktorarbeit befasste sich mit der Analyse von hMutLα-interagierenden Proteinen. Wir glauben, dass die Identifikation von neuen Interaktionspartnern von hMLH1 und hPMS2 essenzielle Informationen über die Rolle dieser beiden Proteine in der MMR und/oder in anderen DNA-reparaturverwandten Mechanismen aufdecken könnte. Zu diesem Zweck nutzten wir die kürzlich entwickelte Zwei-Schritt-Technologie der Tandem-Affinitäts-Reinigung (TAP), welche die Aufreinigung von Interaktoren unter nativen Bedingungen erlaubt und sich, vorallem in Bezug auf Genauigkeit und Spezifität, als ausgezeichnete one-step pulldown-Methode erwiesen hat. Wir transfektierten zwei entweder hMLH1- (293T) oder hPMS2-defiziente (HeLa12) Zell-Linien mit einem Vektor, welcher entweder hMLH1 mit N-terminalem TAP-Anhang oder hPMS2 mit C-terminalem TAP-Anhang exprimierte. Wir konnten zeigen, dass die TAP-gekennzeichneten Konstrukte den MMR-Defekt in der jeweiligen Zell-Linie *in vitro* und *in vivo* korrigierten. Total-Zellextrakte von obig genannten Zell-Linien wurden für die TAP gebraucht, die Eluate wurden auf ein Polyacrylamid-Gel geladen und die Banden ausgeschnitten und mittels Massen-Spektrometrie (MS) analysiert. Wir validierten die Technik durch Detektierung der Köder-Proteine und deren Hauptinteraktions-Partner. So konnte eine Anzahl Proteine identifiziert werden, welche jeweils spezifisch für das jeweilige TAP-Eluat waren. Einige dieser Interaktoren wurden beschrieben, der Grossteil jedoch repräsentierte neue Befunde. Besonders bemerkenswert ist, dass der kürzlich identifizierte FancJ factor BACH1 (BRIP1) spezifisch und in grossen Mengen in unseren pulldowns vorhanden war. Wir konnten diese Interaktion durch unabhängige Techniken bestätigen und untersuchen zur Zeit intensiv die Rolle dieser Inteaktion in der MMR. Da zudem keine hPMS1-defizienten Zell-Linien existieren, um die

Komplementation des Defektes mit einem TAP-gekennzeichneten hPMS1-Konstrukt zu erlauben, führten wir grossangelegte (large-scale) Immunopräzipitationen, gekoppelt an MS-Analysen zur Identifizierung der hPMS1-Interaktionspartner durch. Interessanterweise fanden wir unter den identifizierten Proteinen eine Anzahl von Faktoren, welche zum Ubiquitin-Pfad gehören.

Zusammengefasst kann man sagen, dass wir durch die Anwendung der TAP-Technik und mittels Durchführung von large-scale Immunopräzipitationen zahlreiche Polypeptide identifizierten, welche spezifisch in Komplexen mit hMLH1, hPMS2 und hPMS1 vorhanden sind. Nach Auflistung der identifizierten Proteine entsprechend ihrer biologischen Funktion beginnt nun die Validierung der interessantesten Interaktionen und die Charakterisierung der biologischen Rollen derselben, in der Hoffnung, ein besseres Verständnis des *in vivo*-MMR-Prozesses zu erlangen.

3. Introduction

DNA molecules can be damaged in numerous ways. In addition to misincorporation errors arising during replication, spontaneous damage including deamination, depurination and oxidation, together with exogenous effects of radiation and chemical agents all accumulate in DNA and threaten genomic integrity. Most kinds of DNA damage are harmful and its effects are diverse, ranging from disturbed DNA metabolism to triggering of cell cycle arrest or cell death. Long-term effects include irreversible mutations contributing to oncogenesis. For these reasons, it is essential for the cell to have efficient repair mechanisms able to reduce this damage to a tolerable level. The importance of repair can be seen from the fact that DNA is the only biomolecule that is specifically *repaired* while all others are *replaced*. More than 100 genes participate in various aspects of DNA repair, even in organisms with very small genomes, and many repair pathways are highly conserved during evolution. Although cells from various organisms possess a large number of different types of repair systems, each relatively specific for a certain kind of DNA damage, the repair pathways can be grouped into four broad categories: direct reversal of damage, double-strand break repair, translesion synthesis and excision of damaged region, followed by replacement (reviewed in Hoeijmakers J.H., 2001).

The Direct Reversal (DR) pathway consists of repair proteins that act directly on damaged bases and correct the damage without removing the damaged nucleotide. Typical examples of DR are repair of UV-induced cyclobutane pyrimidine dimers by the enzyme CPD photolyase and the removal of alkyl groups from the O^6-position of guanine by methylguanine-methyltransferase, MGMT.

Double-strand breaks (DSB) are a very disruptive form of DNA damage that, if left unrepaired, can lead to broken chromosomes and cell death whereas, if repaired improperly, to chromosome translocations and cancer. Two independent pathways carry out

the repair of DSBs: homologous recombination (HR) and non-homologous end joining (NHEJ). HR relies on the use of homologous DNA sequences as templates for resynthesis, whereas NHEJ provides a faster, but error-prone pathway of repair of DSBs by joining of ends even if there is not significant sequence similarity between them.

Translesion synthesis is carried out by a class of polymerases that lack exonuclease activity and possess a more flexible active site, which allows them to synthesize DNA through a variety of lesions present in the template molecule. This kind of mechanism should not, strictly speaking, be considered as an example of repair, because the damage is left in the DNA, nevertheless it is an important component of the overall cellular response to DNA damage.

Excision repair is the major DNA repair mechanism. In cases where the damage is present in just one DNA strand, it can be repaired by cutting it out and replacing it with new DNA synthesized using the complementary strand as a template. All organisms employ at least three excision mechanisms: base excision repair (BER), nucleotide excision repair (NER) and mismatch repair (MMR).

BER deals with small modifications of individual bases, often arising spontaneously through oxidation, methylation, depurination or deamination. The specificity of the reaction is given by the DNA glycosylases that recognize a subset of base alterations and cleave the N-glycosylic bond between the base and the deoxyribose, creating thus an abasic site. This step is followed by the nicking of the damaged strand by an endonuclease and nucleotide replacement by a DNA polymerase.

The NER pathway removes DNA lesions that cause a structural deformation of the DNA helix. Repair is achieved trough a damage recognition step (binding of a multi-protein complex at the site of damage), a double incision of the damaged strand on both sides of the

damage and by the subsequent removal of the damage-containing stretch of nucleotides. The resulting gap is then refilled by a DNA polymerase followed by religation.

The MMR system is responsible for the removal of biosynthetic errors. It removes mispairs (non Watson-Crick basepairs) in the newly synthesized strand that were missed by the proofreading activity of the polymerase and thus lowers the mutation rate by a factor of 100-1000 to one error per 10^{10} nucleotides synthesized (Modrich P. and Lahue R., 1996). MMR will be the subject of the following discussion. I will briefly review our knowledge of the mechanism of MMR, the biological function of the proteins involved in MMR and the link between MMR and human cancer.

3.1. MMR in *E. coli*

The function of the *E. coli* MMR system has been well characterized and has served as a paradigm for the eukaryotic MMR. The entire reaction has been reconstituted *in vitro* (Lahue R.S. et al., 1989) and involves three dedicated proteins: MutS, MutL and MutH. The MMR reaction can be divided into three steps: initiation, excision and resynthesis (reviewed in Iyer R.R. et al., 2006). In the first step, the mismatch is detected by a homodimer of MutS. MutS can bind to insertion-deletion loops (IDLs) 1-4nt in length and to all the possible base-base mismatches with the exception of C/C. The crystal structures of MutS from *E.coli* and *T. aquaticus* have been solved (Lamers M.H. et al., 2000; Obmolova G. et al., 2000); the structures are very similar and provide invaluable insight into protein function. MutS appears as a modular protein, consisting of separate domains. It embraces the DNA and dimerizes at the top and bottom, leaving two channels of approximately 30Å and 40Å in the centre with the DNA kinked by 60° and accommodated in the latter (Fig.1).

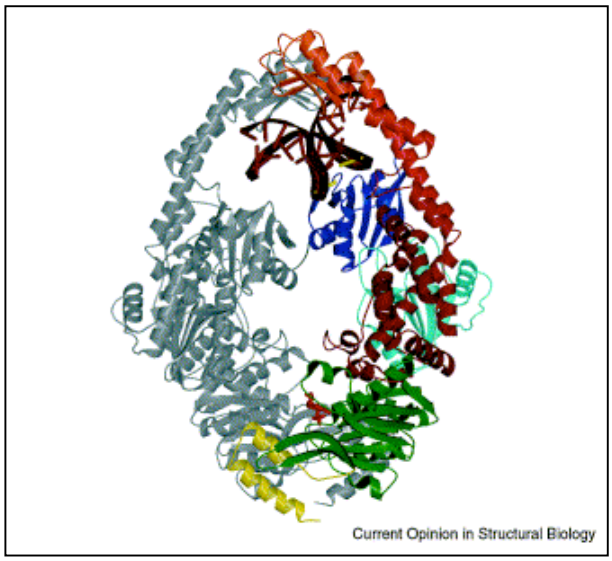

Fig.1 *E. coli* MutS structure. The mismatch-binding monomer is shown in domains: N-terminal mismatch-recognition domain, dark blue; connector domain, light blue; core domain, red; clamp, orange; ATPase domain, green with red ADP; helix-turn-helix domain, yellow. The other monomer is shown in grey. DNA is shown in red, with a yellow mismatch. (Sixma T.K., 2001)

It is notable that even though MutS is a homodimer, only one subunit actually binds the mismatch, although both contact the DNA to form a clamp. This functional asymmetry is an interesting preview of the heterodimers that are found in eukaryotes.

In the presence of ATP and a mismatch, MutS recruits a homodimer of MutL and together they activate MutH. MutH is a latent endonuclease that preferentially cleaves the hemi-methylated *dam* (GATC) sites that transiently exist after a replication fork passes. Thus, in *E. coli*, DNA methylation is used as the strand discrimination signal: the nicking of the unmethylated DNA strand by MutH ensures that MMR targets the repair to the error-containing newly synthesized strand. The nick serves as a point of entry for Ssb (single-strand DNA binding protein) and helicase II, whose loading at the nick is facilitated *via* protein-protein interactions with MutL. A hallmark of MMR is the bidirectionality of the process. The MutH-generated nick can occur either 5' or 3' to the mismatch at distances up to 1kb away. Depending on which side the cleavage occurs, one of several single-strand specific 5'-3' (RecJ or ExoVII) or 3'-5' (ExoI , ExoX or ExoVII) exonucleases (ExoVII supports excision in both directions) degrade the DNA tract between the nicked, hemimethylated site and the mismatch. The resynthesis is then mediated by DNA polymerase III holoenzyme, Ssb and DNA ligase (Fig.2).

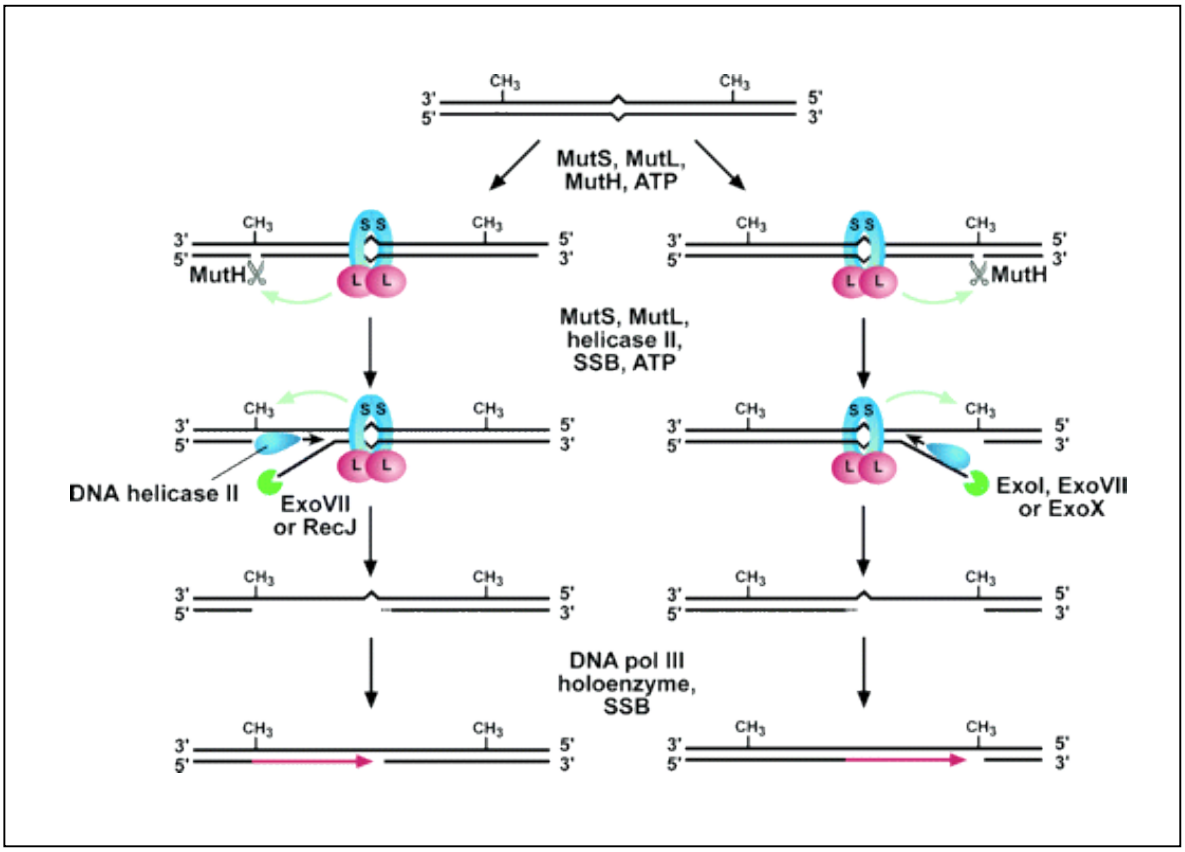

Fig.2 Mechanism of *E.coli* MMR. Although not shown, the ligation step is carried out by DNA ligase after DNA polymerase III holoenzime fills in the gap (Iyer R.R. et al., 2006).

3.2. MMR in eukaryotic cells

Genes encoding homologues of the MutS and MutL bacterial proteins have been identified in several eukaryotes, including yeast, plants, insects, nematodes and mammals. Although the principle repair mechanism is conserved, the eukaryotic MMR system is more complex. In contrast to the situation in prokaryotes, where MutS and MutL function as homodimers, all eukaryotic organisms characterized to date possess multiple MutS and MutL homologues with the active form being heterodimers.

The best characterized eukaryotic MMR system is that of the yeast *S. cerevisae,* where six MutS (*sc*Msh1p-6p) and four MutL homologs (*sc*Mlh1p-3p and *sc*Pms1p) have been identified through a combination of genetic, molecular biological and genome-based techniques (reviewed in Kolodner R.D. and Marsischky G.T., 1999). Of the six MutS

members, *sc*Msh1p appears to solely promote repair and stability of mitochondrial DNA (Reenan R.A.G. and Kolodner R.D., 1992) and although a predicted MSH1-like protein was found in the *A. thaliana* genome, similar homologues have not been found in other organisms.

There are five MutS homologues in humans: hMSH2, hMSH3, hMSH6 that play a role in MMR, and hMSH4 and hMSH5 that are exclusively involved in meiotic recombination. Similarly, four MutL homologues are known: hMLH1, hMLH3, hPMS2, and hPMS1 (the latter two named after the yeast homologues, originally identified in a screening for post-meiotic segregation genes). As mentioned above, the eukaryotic MMR proteins function as heterodimers. Thus, human hMSH2 interacts with hMSH6 or hMSH3 to form the complexes named hMutSα and hMutSβ, respectively. Similarly, human hMLH1 can combine with three different MutL homologues: hPMS2, hPMS1 and hMLH3 to form the complexes hMutLα, hMutLβ or hMutLγ. The various complexes perform specialized functions: hMutSα binds and supports the repair of all base-base mismatches, as well IDLs of 1-4 unpaired nucleotides, although with different efficiency (G/T, A/C, and A/A mismatches and 1nt IDL being the best substrates; reviewed in Marra G. and Jiricny J., 2005). hMutSβ is responsible for the repair of larger IDLs. The activities of the two hMutS heterodimers are thus partially redundant on the repair of small IDLs. Large IDLs, ≥ 10 bases, are repaired by a pathway independent of MMR (Littman S.J. et al., 1999). An interesting feature of the hMutS and hMutL complexes is that although hMSH2 and hMLH1 are stable *per se*, hMSH6, hPMS2 and hPMS1 require the presence of the corresponding interacting partner to be stabilized. The function of the MutL heterodimers is not as well characterized as that of the MutS proteins and will be the subject of further discussion. The most studied heterodimer has been hMutLα that is an essential component

of human MMR and supports the repair initiated by both hMutSα and hMutSα. The identity and functions of *E.coli* and eukaryotic MMR proteins are summarized in Table 1.

***E. coli* protein**	**Function**	**Eukaryotic homologues**	**Function**
MutS	Binds mismatches	MSH2-MSH6 (MutSα)	Repairs single base-base and 1–2 base IDL mismatches
		MSH2-MSH3 (MutSβ)	Repair of some single base IDLs and IDLs $\geq$2 bases Partially redundant with Msh2-Msh6
MutL	Matchmaker that coordinates multiple steps in MMR	MLH1-PMS2 (yPms1) (MutLα)	Matchmaker for coordinating events from mismatch binding by MutS homologs to DNA repair synthesis
		MLH1-MLH2 (hPMS1) (MutLβ)	Function of human heterodimer unknown Suppresses some IDL mutagenesis in yeast
		MLH1-MLH3 (MutLγ)	Suppresses some IDL mutagenesis Participates in meiosis
MutH	Nicks nacsent unmethylated strand at hemimethylated GATC sites	None	
$\gamma-\delta$ Complex	Loads β-clamp onto DNA	RFC complex	Loads PCNA, modulates excision polarity
β-Clamp	Interacts with MutS and may recruit it to mismatches and/or the replication fork Enhances processivity of DNA pol III	PCNA	Interacts with MutS and MutL homologs Recruits MMR proteins to mismatches Increases MM binding specificity of Msh2-Msh6 Participates in excision and probably in signaling Participates in DNA repair synthesis
Helicase II	Loaded onto DNA at nick by MutS and MutL Unwinds DNA to allow excision of ssDNA	None	

(*Continued*)

E. coli protein	Function	Eukaryotic homologues	Function
ExoI ExoX	Perform 3′ to 5′ excision of ssDNA	EXOI (Rth1)	Excision of dsDNA
RecJ ExoVII	Perform 5′ to 3′ excision of ssDNA (also 3′ to 5′ excision by ExoVII)	3′ exo of Pol δ 3′ exo of Pol ε	Excision of ssDNA Synergistic mutator with Exo1 mutant
DNA pol III	Accurate resynthesis of DNA	DNA pol δ	Accurate repair synthesis
Ssb	Participates in excision and DNA synthesis	RPA	Participates in excision and in DNA synthesis
DNA ligase	Seals nicks after completion of DNA synthesis	DNA ligase	Seals nicks after completion of DNA synthesis

[a]Abbreviations are dsDNA, double-stranded DNA; MM. mismatch: PCNA. proliferating cell nuclear antigen; RFC, replication factor C; and ssDNA, single-stranded DNA. RPA, replication protein A

Table 1 *E.coli* and eukaryotic proteins required for MMR (adapted from Kunkel T.A. and Erie D.A., 2005)

3.2.1. Overview of the MMR process

The repair reaction must be targeted to the mismatch-containing newly synthesized strand. MutH, which confers strand specificity in *E. coli*, is not present in eukaryotes, which must therefore discriminate strands in a different way. Given that the *in vitro* MMR reaction is nick-dependent, it was suggested that the gaps between Okazaki fragments on the lagging strand or the free 3' terminus on the leading strand could provide an appropriate strand discrimination signal (Fang W.H. and Modrich P., 1993; Holmes J. et al., 1990; Thomas D.C. et al., 1991). However, although strand discontinuities are sufficient to direct MMR *in vitro*, the natural signal that directs the reaction *in vivo* is still undetermined. Similarly, it is still unclear what couples mismatch recognition by the MutS heterodimer with downstream events. Central to this question is how MSH proteins bound to a mismatch recruit other partners. The C-terminus of all MutS homologues is highly conserved and contains an

ATP-binding site. Several reports show a strict connection between nucleotide binding and DNA binding. Heteroduplex DNA has been shown to increase the rate of ATP hydrolysis by hMutSα several fold (Gradia S. et al., 1999 and 2000). Conversely, the addition of ATP reduces the relative affinity of these proteins for DNA, resulting in an apparent dissociation of hMutSα from the mismatch-containing substrate (Blackwell L.J. et al., 1998; Iaccarino I. et al., 1998; Gradia S. et al., 1997; Hughes M.J. and Jiricny J., 1992). Although ATP binding and hydrolysis is indisputably important in this process, its exact function(s) is still under investigation and several models have been suggested. In the "active translocation" model, based on electron-microscopy studies of *E.coli* MutS (Allen D.J. et al., 1997), it is proposed that ATP promotes bidirectional translocation of hMutSα by drawing flanking DNA toward the protein complex after its initial mismatch binding, yielding a Ω-like loop (Blackwell L.J. et al., 1998). In a second model (the "sliding clamp" model), it is proposed that mismatch recognition by hMutSα provokes an ADP→ATP exchange. The nucleotide switch results in a conformational transition of the protein that allows it to act as a diffusible clamp, and to slide along the DNA in an ATP-hydrolysis independent manner, signalling to additional components of the MMR machinery (Gradia S. et al., 1999). Both models suggest that, upon ATP binding, hMutSα moves away from the mismatch site in search of a strand discrimination signal before activating the repair process. A third model has been proposed more recently on the basis of the crystal structure of bacterial MutS bound to mismatches, which challenges the idea that the proteins leave the mismatch. Based on the sharp kinking of mismatched DNA (Obmolova G. et al., 2000; Lamers M.H. et al., 2000) the "transactivation model" argues that MutS remains in the vicinity of the mismatch and that communication between the mismatch and subsequent repair factors is promoted *via* protein-protein interactions (Junop M.S. et al., 2001). A critical observation in support of this model in bacteria came from the observation that, in the presence of MutL

and ATP, MutS can activate MutH-mediated cleavage *in trans* (Junop M.S. et al., 2001). The fact that human MMR can be initiated despite the presence of blockades between the mismatch and the nick (Wang H. and Hays J.B., 2004) speaks in favor of this model also for eukaryotic MutS proteins. Finally, based on atomic force microscope images and DNA binding assays, a fourth model was suggested, where the mismatch-bound *sc*MutSα homologue serves as a nucleation site for polymerization of a second protein (*i.e. sc*MutLα homologue) along the helix (Hall M.C. et al., 2001). Clearly, additional biochemical, biophysical and structural studies are necessary to further establish the merit of these models and so to clarify the exact mechanism involved in MMR initiation. The proteins involved in the MMR steps of excision and resynthesis have been recently identified. These include exonuclease EXO1 (Tishkoff D.X. et al., 1998; Genschel J. et al., 2002), single-strand DNA binding protein RPA (Lin Y. et al., 1998; Ramilo C. et al., 2002), proliferating cellular nuclear antigen (PCNA) (Umar A. et al., 1996; Gu L. et al., 1998) and DNA polymerase δ (Longley M.J. et al., 1997). An additional replicative DNA polymerase, Pol ε, has been implicated in MMR, but its specific role in the process is still unclear (Pospiech H. and Syvaoja J.E., 2003). The yeast DNA helicase, *sc*Rrm3p, has been postulated to be involved in MMR, based on its interaction with *sc*PCNAp (Schmidt K.H. et al., 2002), but its human equivalent has not been identified.

Identification of these factors made it possible to reconstitute the MMR system *in vitro* from purified proteins and to suggest a model for MMR in human cells (Fig. 3). According to the latest *in vitro* studies (Dzantiev L. et al., 2004; Constantin N. et al., 2005), upon ATP-driven conformational change of hMutSα and recruitment of hMutLα, the ternary complex encounters a strand discontinuity, possibly bound to PCNA.

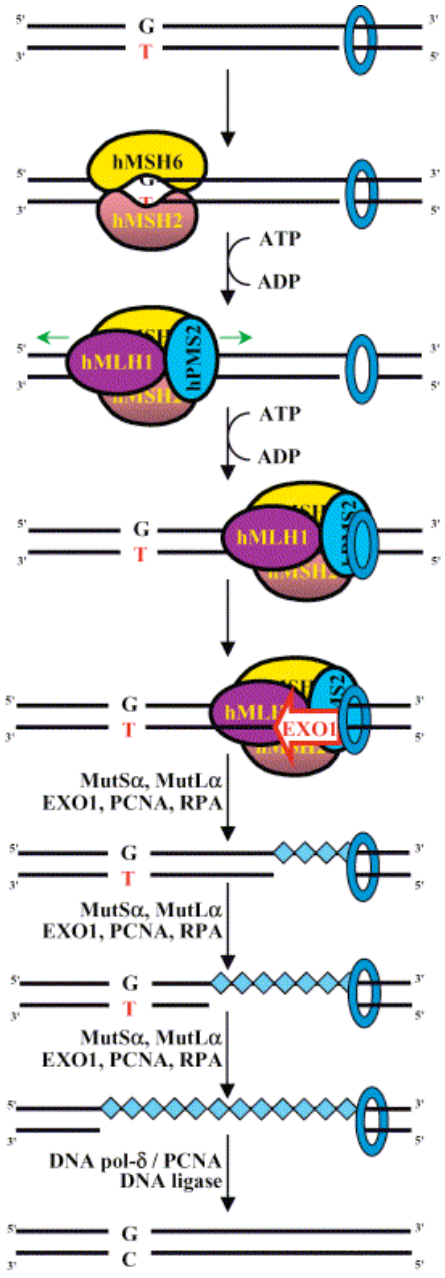

Fig. 3 Schematic representation of human MMR (see text for details). Blue circle: PCNA; blue diamonds: RPA. (Stojic L. et al., 2004)

This event triggers the loading of EXO1 and stimulates its activity so that it starts to degrade the nicked strand in 5'-3' orientation, toward the mismatch. The region of single stranded DNA exposed by EXO1 is stabilized by the binding of RPA. The process continues in the direction of the mismatch through several cycles of binding of EXO1, degradation of several hundreds nucleotides, dissociation of EXO1 followed by its further stimulation by an additional hMutSα/hMutLα/ATP complex, until the degradation passes the mismatch. The repair is then completed with the filling of the gap by the replicative DNA polymerase δ and the sealing of the nick by a yet unidentified ligase. This model accounts for a 5'-3' mismatch–provoked excision due to the 5'-3' activity of EXO1 and although a recent publication shows a bi-directional *in vitro* repair mediated by an additional 3'-5' cryptic activity of EXO1 (Dzantiev L. et al., 2004), more data are necessary to clarify this point. It is however important to note that the situation *in vivo* might be dramatically different and involve several additional factors, with possible functional redundancies.

3.2.2. MutL proteins and MMR

While MutS homologues are responsible for mismatch recognition, the function of MutL homologues is more difficult to characterize. These proteins seem to play an essential role in MMR by coordinating various protein-protein interactions, thereby earning the pseudonym “molecular matchmakers”, but their exact biological role remains enigmatic. Human hMLH1 can form heterodimers with either hPMS2, hPMS1 or hMLH3 to form three complexes designated hMutLα, hMutLβ or hMutLγ. Although a structure is not yet available for full length MutL, crystal structures for the N-terminal 40-kDa of *E.coli* MutL (LN40) and hPMS2 (NhPMS2) in free form and bound to ADP or ATP analogs, as well as for a C-terminal dimerization domain fragments of MutL have been obtained (Ban C. and Yang W., 1998; Ban C. et al., 1999; Guarne A. et al., 2001; Guarne A. et al., 2004). MutL proteins share sequence homology at their N termini over the first 300-400 residues. LN40 bound to ATP is a monomer in solution however, when bound to a non-hydrolysable ADP, it undergoes a large conformational change and forms a dimer. On the basis of sequence and structural analyses, MutL is a member of the GHKL superfamily of ATPases, which includes also DNA gyrase, a type II topoisomerase, Hsp90 and histidine kinase (Dutta R. and Inouye M., 2000). The ATPase activity of MutL and its human homologues is substantially less robust than that of ATP-driven motor proteins, which suggests that ATP is used to modulate conformational changes in the protein that facilitate interactions with other factors and/or DNA. The integrity of the ATPase domain is required for MMR (Rashle M. et al., 2002; Tomer G. et al., 2002). Mutational studies of the ATP binding sites of *sc*MutLα showed that both subunits have intrinsically different ATPase activities both of which are important for MMR (Hall M.C. et al., 2002; Welz-Voegele C. et al., 2002). Thus, similarly to MutS, also MutL proteins are functionally asymmetric and likely bind and hydrolyse ATP in a sequential or alternating manner during MMR. The structure of

NhPMS2 is overall quite similar to that of LN40 and similarly the hPMS2 fragment hydrolyses ATP, although slowly. An interesting difference is that the hPMS2 40-kDa fragment is the first example of a GHKL ATPase active as a monomer (Guarne A. et al., 2001). The C-terminal regions of MutL homologues share little sequence similarity. The C-terminal dimerization domain of MutL has been modelled as a V-shaped dimer with a large cavity that has been postulated to accommodate DNA (Guarne A. et al., 2004).

Similarly to MutS, *E.coli* MutL is a DNA-binding protein with affinity for both single- and double-strand DNA (Junop M.S. et al., 2003), and the MutL ATPase is dramatically activated by ssDNA and to a lesser extent by dsDNA (Spampinato C. and Modrich P. 2000; Ban C. et al., 1999). In contrast, the ATPase activity of hMutLα doesn't respond to DNA, although the N-terminal ATPase of hPMS2 subunit binds DNA with a preference for duplex molecules (Hall M.C. et al., 2001). Also yeast *sc*MutL homologues bind DNA and mutations in the DNA binding domain of *sc*Mlh1p result in a mutator phenotype and loss of heteroduplex repair, suggesting that *sc*Mlh1p-DNA binding is important for MMR (Hall M.C. et al., 2001; Hoffmann E.R. et al., 2003).

As mentioned above, hMLH1 can interact with two additional MutL homologues, hPMS1 and hMLH3. hMutLβ (heterodimer of hMLH1 and hPMS1) does not appear to have a role in MMR *in vitro* (Raschle M. et al., 1999) and other functions of this protein have not been discovered yet. With the exception of hMLH1 (Raschle M. et al., 1999) and hMutSα (Plotz G. et al., 2002), additional proteins interacting with hPMS1 are at the moment not known.

hMLH3, the third hMutL protein able to heterodimerise with hMLH1 in a complex called hMutLγ, was first identified in *S. cerevisiae,* where it binds yeast *sc*Mlh1p (Wang T.F. et al., 1999). Trough mutation analyses it was suggested to be involved in the repair of a subset of IDLs, working in concert with *sc*Msh3p (Harfe B.D. et al., 2000; Flores-Rozas H. and Kolodner R.D., 1998). The human hMLH3 protein was shown to interact with hMLH1

by far-western and mammalian two-hybrid experiments (Lipkin S.M. et al., 2000; Kondo E. et al., 2001). Based on the phenotype of *Mlh3*-/- mice (see chapter 3.2.3.4), a possible role of the mouse Mlh3 in MMR was suggested (Chen P.C. et al., 2005), however the heterodimer hMutLγ was not characterized and its role in MMR was not clear. A better understanding of the role of hMLH3 in human was the aim of the first part of my work that resulted in the first data showing the expression levels of this protein in human cells and its possible role in MMR (Cannavo E. et al., 2005 and *Results*).

3.2.3. MMR defects and cancer

MMR defects are linked to both hereditary and sporadic forms of colorectal cancer.

The hallmark of MMR–deficient tumours is microsatellite instability (MSI), which is manifested by gain or loss in the number of mainly mono- and di-nucleotides repeats. To standardize testing, a set of five short DNA repeats has been recommended for sensitive detection of MSI. If two or more of the five sequences show repeat length variation, the sample is considered MSI positive.

3.2.3.1. Hereditary Non-Polyposis Colon Cancer (HNPCC)

The importance of MMR is underlined by the fact that germline mutations of human *MMR* genes cause susceptibility to HNPCC. HNPCC is the most common form of hereditary colon cancer, accounting for about 5% of all colon cancers (Lynch H.T. and de la Chapelle, 1999; Truninger K. et al., 2005) one of the most common neoplasias in Western populations. HNPCC is a heritable autosomal dominant disease, which is defined, according to the international diagnostic criteria (Amsterdam Criteria I), by the presence of colorectal cancer in at least three family members in two successive generations, with one affected member having been diagnosed at less then 50 years of age (Vasen H.F.A. et al.,

1991). In addition to colon cancer, HNPCC patients have higher risk to develop cancers of the endometrium, and to a lesser extent of the small bowel, urethra and renal pelvis; the diagnostic criteria were later revised to take these extra-colonic cancers into account (Amsterdam Criteria II; Vasen H.F.A. et al., 1999).

Germline mutations in *MMR* genes are detected in up to 70-80% of HNPCC families (Liu B. et al., 1996; Wijnen J. et al., 1997). Individuals with HNPCC carry heterozygous germline mutations in *MMR* genes however, when tumours arise, they have usually lost the second wild-type allele through somatic events. Since the initial identification of HNPCC-linked genes in 1993, HNPCC families have been extensively screened for mutations in *MMR* genes. Mutations of *hMSH2* and *hMLH1* account for about two-thirds of all HNPCC kindreds tested. This percentage reflects the relative importance of the functional role of the products of these genes in MMR, as judged by the fact that they are essential components of all hMutS and hMutL heterodimers. Due to the partial functional redundancy of hMSH6 and hMSH3, germline mutations in hMSH3 have not been found and mutations in hMSH6 are less frequent (~10%) and associated usually with a low degree of MSI. Moreover, these mutations occur often in clinically atypical HNPCC families. Surprisingly, although in a recent study (Truninger K. et al., 2005) the percentage of HNPCC-associated germline mutations in *hPMS2* was reported to be similar to that of *hMSH2*, mutations in *hPMS2* show a low penetrance and occur in atypical HNPCC families. Considering that the heterodimer hMutLα (consisting of hMLH1 and hPMS2) is an essential player in MMR, a clear connection between mutations in *hPMS2* and HNPCC would be expected. One possible explanation is the existence of two additional hMLH1-containing heterodimers in human cells that could account for a partial redundancy in MMR. In the case of hMutLβ, the heterodimer does not seem to participate in MMR *in vitro* (Raschle M. et al., 1999) and

there is no evidence that *hPMS1* is an HNPCC-associated gene, even though the possibility that this gene is implicated in the susceptibility to non-HNPCC cancers cannot be excluded. Although *hMLH3* germline mutations have been described in familial colorectal cancer, the involvement of hMLH3 in tumorigenesis of HNPCC is controversial. *hMLH3* germline mutations were found in families carrying a second *MMR* gene mutated in some cases, whereas other groups reported somatic mutations of the gene as the primary event in HNPCC patients. Similarly, the MSI status of the tumour in case of *hMLH3* mutations is contradictory (Liu H.X. et al., 2003; Hienonen T. et al., 2003; de Jong M.M. et al., 2004; Wu Y. et al., 2001). We could show (Cannavo E. et al., 2005 and *Results*) that hMutLγ (hMLH1 and hMLH3 heterodimer) has a marginal role in MMR *in vitro* and that *hMLH3* can be epigenetically silenced *in vivo* so that a role of hMLH3 in human cancer, possibly in combination with additional factors, cannot be excluded.

Finally, a controversy also exists in terms of whether germline mutations of *hEXO1* are linked to HNPCC. Several germline mutations of *hEXO1* have been reported in HNPCC families, but the same alterations were also identified in normal populations, suggesting that the alterations represent polymorphisms (Wu Y. et al., 2001; Jagmohan-Changur S. et al., 2003). Nevertheless, the creation of the *Exo1*-/- mice (see chapter 3.2.3.4) suggests that a role of *hEXO1* mutations in colon cancer predisposition is possible.

3.2.3.2. Sporadic colorectal cancer

MSI occurs in 15% approximately of sporadic tumours of colorectum and other organs (Boland C.R. et al., 1998). The majority of sporadic colorectal cancers with high MSI are caused by inactivation of *hMLH1*. Whereas in HNPCC the *MMR* genes are inactivated mainly by somatic mutations or loss of heterozygosity, in sporadic hMLH1-deficient colorectal cancers, the inactivation mostly results from gene silencing through promoter

hypermethylation (Kuismanen S.A. et al., 2000). *hMLH1* promoter inactivation seems to be often biallelic, as suggested by studies in cell lines (Veigl M.L. et al., 1998) and it is present already in non-neoplastic precursor lesions of colon cancer, indicating that it is an early event in tumorigenesis (Toyota M. et al., 1999).

3.2.3.3. Tumorigenesis and MMR deficiency

The mutation rates in tumour cells with MMR deficiency are 100-1000 fold higher then in normal cells (Bhattacharyya N.P. et al., 1994). As mentioned above, these mutations occur with especially high frequency in genes containing microsatellites. As numbers of these genes are tumour suppressors, their mutational inactivation is believed to promote cancerogenesis. A selection of these genes is shown in table 2.

Gene	Function	Type of repeat	Proportion of tumors with mutations	Predicted consequence
TGFβRII	Tumor suppressor	$(A)_{10}$	7/7 (100%)	Loss of tumor suppression
			100/111 (90%)	Loss of tumor suppression
MSH3	DNA MMR	$(A)_8$	16/41 (39%)	Enhancement of genomic instability following inactivation
MSH6	DNA MMR	$(C)_8$	12/40 (30%)	Enhancement of genomic instability following inactivation
IGFIIR	Tumor suppressor	$(G)_9$	3/35 (9%)	Loss of tumor suppression
BAX	Promotes apoptosis	$(G)_8$	21/41 (51%)	Loss of pro-apoptotic activity
TCF4	Transcription factor (*Wnt* signaling)	$(A)_9$	19/49 (39%)	Stimulation of transcriptional activity
MBD4	DNA glycosylase, Methyl-CpG binding protein	$(A)_{10}$	10/23 (43%)	Impaired G/T MMR (glycosylase domain lost) Interference with transcriptional repression? (methyl-CpG binding domain retained)
			10/42 (24%)	Loss of function?
PTEN	Tumor suppressor	$(A)_6$ in exon 7, $(A)_6$ in exon 8	6/32 (19%)	Loss of tumor suppression
RIZ	Tumor suppressor	$(A)_8$, $(A)_9$ in exon 8	9/24 (37.5%)	Loss of tumor suppression
			14/51 (26%)	Loss of tumor suppression
AXIN2	*Wnt* signaling	Four mono-nucleotide repeats in exon 7	11/45 (24%)	Induction of *TCF*-dependent transcription

Table 2 Selection of target genes for frameshift mutations in MSI colon cancers (Adapted from Peltomaki P., 2001)

Moreover, functional MMR was shown to suppress recombination between homeologous sequences. As a result, MMR-deficient cells might have higher rates of gene conversion, which might expose tumour suppressor genes to loss of heterozygosity (Ciotta C. et al.,

1999). Finally, MMR-deficient cells fail to promote apoptosis upon exposure to certain genotoxic agents (see chapter 3.3.1). This might allow cells with damaged DNA to survive and potentially give rise to tumours, rather then die (Bardelli A. et al., 2001).

3.2.3.4. Mouse models of human MMR defects

The development of mouse cell lines with targeted inactivation of all known *MMR* genes has critically helped the understanding of the relationship between MMR and tumorigenesis in HNPCC (reviewed in Wei K. et al., 2002). In general, the phenotype of *MMR* mutant mice correlates well with mutation analysis in HNPCC patients and repair defects of cell lines lacking the corresponding protein. A major difference is that, unlike HNPCC patients, only homozygous and not heterozygous mice develop tumours. This feature is most likely due to the short life span and small size of the mice, which make the somatic loss of the second allele less probable. The spectrum of tumours in these mice includes mainly gastrointestinal (GI) and skin cancers, with a particular susceptibility to lymphomas. In addition, MMR deficient mice do not develop colorectal cancer, but rather tumours of the small intestine. The phenotype of the MMR deficient knock-out mice is shown in table 4.

Gene	MSI	Tumor	Fertility
MSH2	Yes	Lymphoma, GI, skin, and other tumors	Yes
MSH3	Yes	GI tumors	Yes
MSH6	Low instability in dinucleotide repeats	Lymphoma, GI and other tumors	Yes
MLH1	Yes	Lymphoma, GI, skin, and other tumors	No
PMS1	Mononucleotide repeats only	None	Yes
PMS2	Yes	Lymphoma and sarcoma	Male only
MLH3	Yes	Lymphoma, GI	No
EXO1	Mononucleotide repeats only	Lymphoma	No

Table 4: Phenotype of MMR-deficient knock-out mice (Adapted from Li G.M., 2003 and Chen P.C. et al., 2005)

A striking feature of the *Mlh1*, *Pms2* (males only) and *Mlh3* knock-out mice is sterility, which does not appear in *Msh2*- and *Msh6*-deficient animals. This implies additional

functions for MutL homologue proteins in meiosis, which will be discussed in chapter 3.3.2.

Moreover, the tumour spectrum of *Mlh1*-/- animals differs from that of *Pms2*-/- mice, suggesting that other proteins might have redundant roles with *Mlh1*. Indeed Chen P.C. et al. (2005) recently reported that *Mlh3*-/- animals develop GI cancers and lymphomas, albeit at a later age.

3.3. Additional roles of MMR

3.3.1. MMR deficiency and drug resistance

While MMR is well characterized for its role in correcting replication errors, other important roles of MMR proteins have been discovered. Most notably, MMR was shown to mediate cell killing upon treatment with several genotoxic agents (reviewed in Stojic L. et al., 2004). MMR-deficient cells have been shown to be around 100-fold more resistant to methylating agents (S_NI-type) and two to three folds to cisplatin, than cells with functional MMR.

Upon treatment with low doses of S_NI -type alkylating agents such as MNNG (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine) or its close relative Temozolomide used in cancer chemotherapy, MMR-proficient cells usually undergo growth arrest at the G_2-phase of the cell cycle, which is followed by apoptosis. MMR-deficient cells treated with the same doses do not respond to the treatment, thus the apoptotic response only occurs in the presence of functional MMR (reviewed in Stojic L. et al., 2004). The molecular events involved in the MMR-dependent apoptotic response have not been established yet, however, two models have been proposed.

S_NI -type alkylating agents give rise to O^6-methylguanine (O^6-MeG), which templates thymidine during replication. If demethylation is not carried out by the methyltransferase

MGMT, the MMR machinery will recognise the O^6-MeG/T mismatch. However, because MMR is always targeted to the newly synthesized strand, adducts in the template strand cannot be removed and thus the unusual base pair re-forms upon DNA resynthesis. As a result, the repair cycle might be perpetually reinitiated. Such a futile repair cycle may signal cells to switch on the apoptotic machinery (Karran P., 2001). A second model suggests the possibility that MMR proteins might act as damage sensors and directly transduce the damage signal to downstream components (Fishel R., 1999).

As mentioned above, MMR-deficient cells show a two to three fold higher resistance also to cisplatin compared to MMR-proficient cells. Although this difference is well documented (reviewed in Stojic L. et al., 2004), the role of MMR in the sensitivity to cisplatin is less critical than in the case of alkylating agents. It is clear that the lesions produced by cisplatin (mainly intra- and inter-strand cross-links) cannot possibly be mistaken for a simple mismatch by the MMR machinery; this kind of lesions would rather block the progression of the replication fork. The mechanism underlying the MMR-dependent response to cisplatin must therefore differ from that controlling the response to alkylating agents. Clearly, more experiments are needed to clarify this point.

The role of MMR in the response to other DNA damaging agents, such as ionizing radiation, UV radiation, topoisomerase inhibitors or interstrand cross-linking agents is more controversial. Although a differential response to those agents was reported to be MMR-dependent, when those agents were tested using strictly isogenic MMR-proficient and deficient cells, no differences were observed (Papouoli E. et al., 2004 and references within).

3.3.2. Role of MMR proteins in meiosis

Some MMR proteins have meiosis-specific functions that appear to operate independently of mismatch binding/repair. In meiosis, homologous recombination serves to promote genetic diversity and to create a physical link between homologous chromosomes that is crucial for assuring their proper segregation during the first meiotic division. A subset of MMR proteins is required for the formation of crossovers and the proper segregation of homologues (reviewed in Kolas N.K. and Cohen P.E., 2004; Hoffmann E.R. and Borts R.H., 2004). The MutS homologues MSH4 and MSH5 (that interact to form a heterodimer) appear to have completely lost the ability to participate in MMR instead, the loss of either is associated with approximately 50% reduction in meiotic crossing over (Hollingsworth N.M. et al., 1995; Ross-Macdonald P. et al., 1994). Mice deleted for *Msh4* or *Msh5* are sterile, show asynapsis of homologous chromosomes but do not develop tumours (Edelmann W. et al., 1999; de Vries S.S. et al., 1999; Kneitz B. et al., 2000).

MutL homologues MLH1, MLH3 and PMS2 appear also to have a role in meiotic recombination, in addition to their function in MMR. Knock-out mice for *Mlh1* are sterile and the complex Mlh1-Mlh3 is formed during murine meiosis (Kolas N.K. et al., 2005). In addition, Mlh3 interacts with Msh4 in mammalian meiotic cells (Santucci-Darmanin S. et al., 2002), suggesting that MLH1-MLH3 might act during meiosis together with MSH4-MSH5 (Kolas N.K. et al., 2005). As would be expected if Mlh3 functioned together with Mlh1, *Mlh3*-/- mice are infertile. Mlh3 seems to be required for the localization of Mlh1 to meiotic chromosomes and for the formation of late recombination nodules (Lipkin S.M. et al., 2002). The absence of Pms2 in mice causes abnormal chromosome synapsis and sterility but, intriguingly, only in males (Baker S.M. et al., 1995). The data available up to date point to a role of PMS2 in mammalian meiosis (Kolas N.K. et al., 2005), but the exact function and mechanism remains unclear. Finally, mice carrying an inactivating mutation in

Exo1 are infertile (Wei K. et al., 2003), but the role of this protein in crossing over remains elusive.

3.3.3. Further roles of MMR proteins

MMR proteins participate in several other mechanisms that involve heterodouplex formation and/or recombination. For instance they have important anti-recombination functions. MutS and MutL proteins suppress recombination between quasi-homologous sequences, so called homeologous recombination. The anti-recombination activity of MMR in prokaryotes is thought to constitute a barrier to DNA transfer between different species (Vulic M. et al., 1997). Studies in yeast and mammalian cells have demonstrated similar functions of the eukaryotic MutS and MutL proteins (reviewed in Surtees J.A. et al., 2004). Studies in *S.cerevisae* have identified a specialized role of *sc*Msh2p-*sc*Msh3p in homologous recombination and a type of recombination known as single strand annealing (SSA) (reviewed in Paques F. and Haber J.E., 1999). In homologous recombination, a single-stranded tail with a 3'-end invades a homologous duplex DNA molecule, and the invading 3'-end is then used to prime DNA synthesis. If the 3'-end itself is not homologous to the invaded duplex, the nonhomologous segment must be removed before DNA synthesis can be initiated. In the SSA recombination pathway, a double strand break between directly repeated sequences is acted on by a 5'-3' exonuclease to yield long tails with 3' ends. Genetic studies have demonstrated that *sc*Msh2p and *sc*Msh3p cooperate with *sc*Rad1p-*sc*Rad10p endonuclease to effect the removal of nonhomologous 3'-tails generated during mitotic homologous recombination and SSA (Saparbaev M. et al., 1996; Sugawara N. et al., 1997).

The recombinational functions of MMR seem also to be responsible for the emerging role of MMR proteins in telomere elongation by a telomerase-independent recombination-based

mechanism, called ALT (Alternative Lengthening of Telomeres; Nguyen B. et al., 2004 and references within). The precise role of MMR defect for the engagement of this ALT telomere elongation remains unclear, although work in yeast suggest that a hyper-recombinogenic phenotype, which is associated with the MMR defect, might facilitate ALT engagement (Rizki A. et al., 2001).

Paradoxically, some MMR proteins also participate in specialized processes that destabilize genetic information. An example is the expansion of trinucleotide repeat sequences that underlie a number of neurodegenerative diseases, such as myotonic dystrophy, Huntington's disease, fragile X-syndrome and Friedreich's ataxia (Wells R.D. et al., 1998). MMR proteins seem to actively contribute to the formation of large expansions, maybe in a replication–independent manner. In particular, a specific role of hMSH2 and hMSH3 in this pathway has been postulated, but the molecular mechanisms responsible for the expansion remains to be established (Owen B.A. et al., 2005).

Finally, two examples where MMR proteins alter genetic information in a positive way are somatic hypermutation (SHM) and class-switch recombination (CSR) (reviewed in Martin A. and Scharff M., 2002). These processes occur in B cells and are required for the development of a normal and highly diverse repertoire of immunoglobulin genes. There are different postulated roles for MMR in SHM and CRS, but additional data are needed to draw any conclusions at the moment.

3.4. References

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4. Results

The results are presented as published papers or manuscripts.

4.1
Expression of the MutL homologue hMLH3 in human cells and its role in DNA mismatch repair

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Expression of the MutL Homologue hMLH3 in Human Cells and its Role in DNA Mismatch Repair

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Abstract

The human mismatch repair (MMR) proteins hMLH1 and hPMS2 function in MMR as a heterodimer. Cells lacking either protein have a strong mutator phenotype and display microsatellite instability, yet mutations in the *hMLH1* gene account for ~50% of hereditary nonpolyposis colon cancer families, whereas *hPMS2* mutations are substantially less frequent and less penetrant. Similarly, in the mouse model, *Mlh1*$^{-/-}$ animals are highly cancer prone and present with gastrointestinal tumors at an early age, whereas *Pms2*$^{-/-}$ mice succumb to cancer much later in life and do not present with gastrointestinal tumors. This evidence suggested that MLH1 might functionally interact with another MutL homologue, which compensates, at least in part, for a deficiency in PMS2. Sterility of *Mlh1*$^{-/-}$, *Pms2*$^{-/-}$, and *Mlh3*$^{-/-}$ mice implicated the Mlh1/Pms2 and Mlh1/Mlh3 heterodimers in meiotic recombination. We now show that the hMLH1/hMLH3 heterodimer, hMutLγ, can also assist in the repair of base-base mismatches and single extrahelical nucleotides *in vitro*. Analysis of hMLH3 expression in colon cancer cell lines indicated that the protein levels vary substantially and independently of hMLH1. If hMLH3 participates in MMR *in vivo*, its partial redundancy with hPMS2, coupled with the fluctuating expression levels of hMLH3, may help explain the low penetrance of *hPMS2* mutations in hereditary nonpolyposis colon cancer families. (Cancer Res 2005; 65(23): 10759-66)

Introduction

Mismatch repair (MMR) proteins are a highly conserved group of polypeptides that play key roles in the correction of mispairs arising during DNA replication. They also prevent recombination between nonidentical sequences and participate in the signaling of certain types of DNA damage. The importance of MMR proteins in the maintenance of genomic integrity is underscored by the finding that germ line mutations in *MMR* genes predispose to hereditary nonpolyposis colon cancer, a common familial cancer predisposition syndrome (reviewed in refs. 1, 2). The principal MMR players in human cells are homologues of the bacterial MutS and MutL proteins. hMutSα, a heterodimer of the MutS homologues hMSH2 and hMSH6, binds base-base mismatches and small insertion/deletion loops, whereas hMutSβ (a heterodimer of hMSH2 and hMSH3) binds only insertion/deletion loops. This *in vitro* evidence could be corroborated by analysis of the phenotypes of MMR-deficient cells: Those lacking hMSH2 are fully MMR deficient and display a mutator phenotype and microsatellite instability that is consistent with the loss of repair of both base-base mismatches and insertion/deletion loops. Cells lacking hMSH6 retain a strong mutator phenotype but their microsatellite instability is limited to mononucleotide repeats due to the functional redundancy with hMutSβ in insertion/deletion loop repair. This situation is mirrored in hereditary nonpolyposis colon cancer families, where the penetrance of *hMSH2* mutations is substantially higher than that of alterations in the *hMSH6* locus (reviewed in ref. 2).

Whereas it is generally accepted that hMutSα and hMutSβ are the mismatch recognition factors that initiate MMR (reviewed in ref. 3), the function of the MutL homologues remains speculative. The human genome contains numerous genes that have significant sequence homology to *mutL* and to yeast MutL homologue and postmeiotic segregation genes; however, to date, only hMutLα, a heterodimer of hMLH1 and hPMS2, could be shown to be involved in MMR. Correspondingly, hMLH1- or hPMS2-deficient cells have a strong mutator phenotype and high microsatellite instability (reviewed in ref. 1). In *in vitro* studies, hMutLα could be shown to associate with hMutSα on a mismatch-containing substrate (4) and was suggested to act as a "molecular matchmaker" between these protein complexes and the downstream effectors of repair (reviewed in ref. 3). hMutLβ, a heterodimer of hMLH1 and hPMS1, has been biochemically characterized but could not be shown to participate in MMR *in vitro* (5). This finding was substantiated by *in vivo* evidence: Mice carrying a disruption in the *Pms1* gene display neither microsatellite instability nor cancer predisposition (6). hMLH3 was identified through its interaction with hMLH1 on Far Western blots (7); however, this heterodimer, hMutLγ, has not been biochemically characterized and its role in mammalian MMR has not been established. *MLH3* was first identified in *Saccharomyces cerevisiae* and its gene product, scMlh3p, was shown to bind scMlh1p (8, 9) and to be involved in meiotic recombination (reviewed in refs. 10, 11). As *mlh3* mutants display a mutator phenotype similar to that of *msh3*-deficient strains (8, 12), it was suggested that the two polypeptides are involved in the repair of a subset of insertion/deletion loops. hMLH3 seems to be involved in meiotic recombination (13, 14) and the same is true for the murine Mlh3 (14). As both Mlh1- and Mlh3-deficient mice are sterile (reviewed in refs. 10, 11), it was suggested that the two polypeptides function together. However, unlike *Mlh1*$^{-/-}$ animals (6), *Mlh3*$^{-/-}$ mice did not succumb to cancer in the first 9 months of life (15). The roles of the various MMR factors and the phenotypes of mice with defects in *MMR* genes are listed in Table 1.

The involvement of MutL homologue malfunctions in human cancer is not as clear cut as in the case of the MutS homologues. Mutations in *hMLH1* predominate in hereditary nonpolyposis

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Table 1. Overview of mammalian MutS and MutL homologues and their roles in MMR

Heterodimer	Components	MMR role	Phenotype of knockout mice
hMutSα	hMSH2	Repair of base-base mismatches and small loops	Lymphomas, gastrointestinal, skin, and other tumors
	hMSH6		Lymphomas, gastrointestinal, and other tumors
hMutSβ	hMSH2	Repair of loops	Lymphomas, gastrointestinal, skin, and other tumors
	hMSH3		Gastrointestinal tumors
hMutLα	hMLH1	Repair of all MMR substrates	Lymphomas, gastrointestinal, skin and other tumors; sterility
	hPMS2		Lymphomas, sarcomas; male sterility
hMutLβ	hMLH1	?	Lymphomas, gastrointestinal, skin, and other tumors
	hPMS1		No phenotype
hMutLγ	hMLH1	?	Lymphomas, gastrointestinal, skin, and other tumors
	hMLH3		Sterility

colon cancer, accounting for nearly 50% of all known germ line *MMR* gene mutations (2). Surprisingly, no germ line mutations have been found in *hPMS1* or *hPMS2*, which was unexpected, given the key role of the latter protein in MMR. Recent immunohistochemical analysis of 1,048 unselected colon tumors revealed the lack of hPMS2 in ~1.5%, a proportion similar to that of MSH2-deficient cancers (16). Genetic analysis identified germ line mutations in *hPMS2* in a number of these patients and it is likely that the remainder will also be linked to genetic alterations once the problems associated with sequencing of the *hPMS2* locus are overcome (there are ~20 *hPMS2* pseudogenes on chromosome 7, which interfere with DNA sequencing). However, these patients do not belong to typical hereditary nonpolyposis colon cancer families and the penetrance of these mutations seems to be very low. One possible explanation for this finding is that the defect in hPMS2 is partially compensated for by another MutL homologue, such as hMLH3. Germ line *hMLH3* missense and frameshift mutations have been described in familial colorectal cancer cases but the implication of these alterations in carcinogenesis is ambiguous. In some cases, the mutation in *hMLH3* was identified in families carrying a second *MMR* gene mutation, whereas no mutations in the other *MMR* genes could be identified in other cases (17–19). A similar discrepancy applies also to the microsatellite instability status of the tumors (17, 20). The role of hMLH3 in MMR and of *hMLH3* mutations in cancer thus remains open to question. In an attempt to provide answers to these questions, we examined the role of hMLH3 in MMR *in vitro*.

Materials and Methods

cDNA Vectors

pFastBac1-His$_6$-hMLH3. The cDNA of *hMLH3* (Swiss-Prot entry Q9UHC1) was used as template for a PCR reaction where $(His)_6$ tag was added at the NH_2 terminus of *hMLH3* using the primers hMLH3fo1 (5′-CGCGGATCCACCATGTCGTACTACCATCACCATCACCATCACG-ATTACGATATCCCAACGACCGAAAACCTGTATTTTCAGGGCATCAAGTG-CTTGTCAGTTGAAG-3′) and hMLH3re1 (5′-ATTTGCCTACTGGTGG-GACC-3′). The fragment was then cleaved with *Bam*H1 and *Pfl*M1 and cloned between the corresponding sites in pFastBac1 (Invitrogen, San Diego, CA).

***pTXB1-hMLH3* (amino acids 961-1,453).** The COOH-terminal part of *hMLH3* cDNA coding for amino acids 961 to 1,453 was amplified by PCR from pFastBac1-His_6-hMLH3 using the primers fMLH3-Ct (5′-GG-GAATTCCATATGGAGAACTGTGTGATATCAGAAACTC-3′) and rMLH3-Ct (5′-AAGGCCGCTCTTCCGCACATTGGTGGCTCACAGGGAGGCATG-3′). The PCR product was subcloned between the *Nde*I/*Sap*I sites of pTXB1 (New England Biolabs, Beverly, MA).

Expression of hMutLγ

The Bac-to-Bac baculovirus expression system (Life Technologies, Gaithersburg, MD) was used according to the instructions of the manufacturer. *Spodoptera frugiperda* Sf9 cells (2×10^8; Life Technologies) were infected with either a single recombinant baculovirus or with a combination of two viruses at a multiplicity of infection of 10. Cells were harvested 72 hours after infection and total extracts were prepared as described (21). Partial purification of hMutLγ from Sf9 extracts was done using Ni-NTA agarose (Qiagen, Hilden, Germany), and the QIAexpressionist system was used according to the instructions of the manufacturer using 5 mL of 50% Ni-NTA slurry per 100 mg of protein extract.

hMutLγ was expressed also in bacteria using a bicistronic vector *pET11b-His$_6$-hMLH3/MLH1* (cloning information on request) in the BL21 strain of *Escherichia coli*. After induction of expression at 37°C for 4 hours with 0.4 mmol/L isopropyl-β-D-thiogalactopyranoside, the heterodimer was expressed but was insoluble. Nevertheless, the protein could be used to quantify the relative abundance of hMLH3 in HeLa cells.

hMLH3 Antibody Production and Purification

The COOH-terminal polypeptide of hMLH3 (amino acids 961-1,453) was expressed using the Impact-CN-System (New England Biolabs) in BL21 *E. coli* transformed with *pTXB1-hMLH3* (amino acids 961-1,453). The peptide was purified using fast protein liquid chromatography on a MiniQ 4.6/50 PE column (Amersham Pharmacia, Uppsala, Sweden) and used to immunize rabbits at Eurogentec (Seraing, Belgium). The rabbit polyclonal antibody was then affinity-purified using the COOH-terminal polypeptide immobilized on a nitrocellulose membrane. In brief, 100 μg of the purified polypeptide were blotted onto a nitrocellulose membrane by standard electrophoretic transfer, visualized by Ponceau S staining, and the corresponding band was cut out. The membrane was blocked with 5% nonfat dry milk in TBST [20 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, and 0.1% Tween 20] for 60 minutes, incubated with 700 μL of the polyclonal antibody for 4 hours at 4°C, and washed thrice with TBST for 15 minutes. The membrane was then cut into small pieces (1×0.5 cm) and the antibody was eluted from the membrane by incubation for 20 minutes at room temperature in 0.1 mol/L glycine (pH 2.5). The supernatant was collected and the pH was neutralized by an equal volume of 1 mol/L Tris-HCl (pH 8.0). The purified antibody was stored at −20°C in 50% glycerol.

It was used to perform all the experiments described in this study except for the immunoprecipitation of hMLH3 from human cell extracts.

Human Cell Lines and Preparation of Cell Extracts

All the colon cancer cell lines, HEK293, and HeLa cell lines used in this study were obtained from the cell line repository of Cancer Network Zurich. The hPMS2-deficient cell lines HeLa clone 12 (22) and Hec-1A (23) were kindly provided by Dr. Margherita Bignami (ISS, Rome, Italy). The cell line HEK293T was derived from HEK293 by immortalization with adenovirus 5 DNA and transfection with SV40 large T antigen (24). The *hMLH1* gene in this cell line is epigenetically silenced by promoter hypermethylation (25). The 293T Lα cell line was developed in our laboratory (26). In these cells, the *hMLH1* c-DNA was stably integrated under the control of the tetracycline response promoter

using the Tet-Off system (Clontech, Palo Alto, CA). In the absence of doxycycline, these cells express hMLH1 and are MMR proficient. All the cell lines were cultured at 37°C in a 5% CO_2–humidified atmosphere and maintained in the appropriate media. Whole cell extracts from these cell lines were prepared as described (26) without modifications. The origin and MMR status of the cell lines used in this study is listed in Table 2.

Western Blot Analysis

Western blots were done as previously described (26) using the following primary antibodies: our rabbit polyclonal anti-hMLH3 (1:400), anti-hMLH1 and anti-hPMS2 from BD PharMingen (San Diego, CA) (1:4,000 and 1:1,000, respectively), and anti-β-tubulin (1:2,000; Santa Cruz Biotechnology, Santa Cruz, CA).

Coimmunoprecipitation Analysis of hMLH1 and hMLH3

HeLa whole cell extract (1 mg) was incubated in a total volume of 500 μL in NP40 Lysis Buffer [50 mmol/L Tris-HCl (pH 8.0), 125 mmol/L NaCl, 1% NP40, 2 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 1× complete protease inhibitory cocktail (Roche Molecular Biochemicals, Basel, Switzerland)] for 3 hours at 4°C with the anti-hMLH1 (6 μg; BD PharMingen) or anti-hMLH3 (10 μg; Santa Cruz Biotechnology) antibodies. The immunoprecipitates were captured by incubation for 30 minutes at 4°C with 50 μL of 50% slurry of Protein A/G PLUS agarose (Santa Cruz Biotechnology). The agarose beads were then washed thrice with cold NP40 Lysis Buffer and the proteins were eluted with SDS sample buffer and subjected to Western blot analysis. Control experiments were done either in the absence of antibody or in the presence of 25 units of Benzonase (Merck, Whitehouse Station, NJ).

Analysis of the *hMLH3* Promoter and Treatment of Cells with 5-Aza-2′-deoxycytidine

The *hMLH3* 5′ flanking region was analyzed for CpG content with the CpG plot software of the European Bioinformatics Institute (http://www.ebi.ac.uk/emboss/cpgplot/) and its methylation status was evaluated with methylation-specific PCR as described previously (16). Primer sequences for the unmethylated reactions were 5′-GTTGTGTGTAGTTTTT-GGAGTTG-3′ (sense) and 5′-CTCCCAACACCTAAAACTAACA-3′ (antisense), which amplified a 229 bp product. The methylation-specific primers were 5′-CGCGTAGTTTTCGGAGTC-3′ (sense) and 5′ CTAAAACTAACGAAACG-CACG 3′ (antisense), which amplified a 205 bp product. The PCR conditions are available on request.

To reactivate the expression of *hMLH1* and *hMLH3*, $2,5 \times 10^5$ HEK293T cells were seeded on a 78 cm^2 dish on day 0 and treated with 3 μg/mL of 5-aza-2′-deoxycytidine (Fluka, Buchs, Switzerland) on days 2 and 5. The medium was changed 24 hours after each addition of the drug and the cells were harvested on day 8.

Microarray Experiments

Microarray experiments were done as described previously (27). Gray columns in the graphs represent mRNA levels based on raw signals detected in the corresponding cell lines with the Affymetrix HG-U133A microarray.

Mismatch Repair Assays

The assays were done as described previously (28, 29).

Results

Expression of hMutLγ in Sf9 cells and production of anti-hMLH3 antibody. To produce the recombinant hMLH3 and hMutLγ factors, *S. frugiperda* Sf9 cells were infected with baculoviruses carrying cDNAs encoding *hMLH1* and/or *hMLH3*. Infection of Sf9 cells with the *hMLH3* virus alone yielded the protein in an amount that was hardly detectable by Western blotting. The amount of expressed protein was significantly increased when the cells were coinfected with both *hMLH1* and *hMLH3* vectors (Fig. 1*A*), suggesting that the presence of hMLH1 is necessary for the stabilization of hMLH3 in Sf9 cells. This is reminiscent of hMSH6 and hPMS2, both of which require their heterodimeric partners (hMSH2 and hMLH1, respectively) for stability. However, the amount of the recombinant heterodimer obtained was too low

Table 2. Characteristics of the human cell lines used in this study

Cell lines	Origin	MMR status*	MMR protein defect †	Genetic complementation
293	Embryonic kidney epithelium	+		
293T	Embryonic kidney epithelium	−	**hMLH1**, hPMS2, **hMLH3**	
293T Lα+	Embryonic kidney epithelium	+	**hMLH3**	*hMLH1* cDNA
CaCo2	Colon carcinoma	+		
CO115	Colon carcinoma	−	**hMLH1**, hPMS2	
Colo741	Colon carcinoma	+		
CX-1	Colon carcinoma	+		
GP5D	Colon carcinoma	−	**hMSH2**, hMSH6, hMSH3, **hMLH3**	
HCT116	Colon carcinoma	−	**hMLH1**, hPMS2, hMSH3	
HCT116+Ch.3	Colon carcinoma	+	hMSH3	Chromosome 3
HT29	Colon carcinoma	+		
Hec1A	Endometrial adenocarcinoma	−	**hPMS2, hMSH6**	
Hec1A+Ch.7	Endometrial adenocarcinoma	−	**hMSH6**	Chromosome 7
HeLa	Cervical carcinoma	+		
HeLa clone 12	Cervical carcinoma	−	**hPMS2**	
LS411	Colon carcinoma	−	**hMLH1**, hPMS2	
SW48	Colon carcinoma	−	**hMLH1**, hPMS2	
SW480	Colon carcinoma	+		
SW837	Colon carcinoma	+		

*+, MMR proficient; −, MMR deficient (these cell lines are unable to repair both base-base mismatches and insertion/deletion loops, with the exception of Hec1A+Ch.7, which is able to repair insertion/deletion loops).
†The primary alteration of MMR protein expression is reported in bold. Lack of hMLH1 or hMSH2 lead to proteolytic degradation of hPMS2, or hMSH6 and hMSH3, respectively. The *hMSH3* gene in HCT116 cells is mutated as a consequence of the MMR defect. The hMLH3 alterations are those described in this study; other alterations have been reported elsewhere (see text).

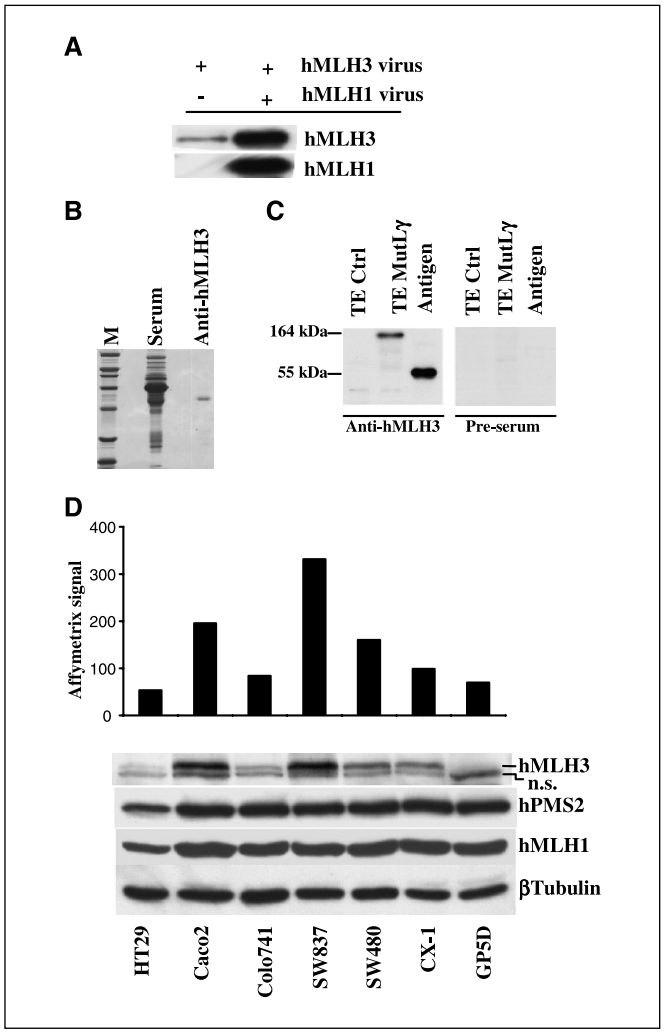

Figure 1. Expression of hMutLγ in Sf9 cells, anti-hMLH3 antibody specificity, and endogenous levels of hMLH3 in colon cancer cell lines. *A,* expression of hMLH3 in Sf9 cells infected either with a baculovirus vector expressing hMLH3 or with a mixture of hMLH1- and hMLH3-expressing viruses. This Western blot shows that hMLH3 is stabilized in this system by hMLH1. *B,* Coomassie blue staining of rabbit polyclonal anti-hMLH3 serum before (*Serum*) and after (*Anti-hMLH3*) affinity purification. *C,* Western blot analysis of whole cell extracts of Sf9 cells uninfected (*TE Ctrl*, 2 μg) or coinfected with the hMLH1/hMLH3 baculoviruses (*TE MutLγ*, 2 μg). The recombinant polypeptide (amino acids 961-1,453) used for the generation of the antibody was loaded in the third lane (1 ng). *Anti-hMLH3,* affinity-purified serum used at 1:400 dilution; *Pre-serum,* preimmune serum used at the same dilution. *D,* microarray analysis of mRNA expression levels (*top*) and Western blot analysis of protein levels (*bottom*) of hMLH3 in a series of colon cancer cell lines (50 μg of whole cell extract per lane); *n.s.,* nonspecific band detected by the anti-hMLH3 antibody in human cell extracts.

to permit extensive purification. The reasons underlying the low levels of expression are unknown at this time, but it is possible that high amounts of the full-length protein may be toxic (7).

Commercially available antibodies could detect the recombinant hMLH3 protein on Western blots but failed to detect the endogenous protein in all the human cell lines used in this study (data not shown). Therefore, we raised our own polyclonal rabbit antiserum, directed against the COOH terminus of hMLH3, which contains the hMLH1-interacting domain. The affinity-purified antibody (Fig. 1*B*) detected a band of the expected size (~160 kDa) in Sf9 lysates infected with the *hMLH1* and *hMLH3* vectors, whereas no signal was visible when we probed lysates of uninfected cells (Fig. 1*C*). The purified antibody was then tested using extracts of various human colon cancer cell lines. The antibody highlighted a double band migrating at the expected size of hMLH3 (Fig. 1*D, bottom*). As the faster migrating band was also observed in Western blots done with the preimmune serum (data not shown), and as the abundance of the slower-migrating band correlated with *hMLH3* mRNA expression levels in the same cell lines (Fig. 1*D, top*), we concluded that the latter is the specific band. As shown in Fig. 1*D*, the levels of hMLH3 fluctuate significantly in the tested cells lines and seem to be independent of the amount of hMLH1 and hPMS2 expressed in the same cells.

Relative abundance of hMLH3 in human cells and its interaction with hMLH1. Given that hMLH3, hPMS2, and hPMS1 interact with the same region of hMLH1 (30), we wanted to ask whether the relative abundance of the three different heterodimers can be correlated with the phenotype of the cells. Therefore, we did semiquantitative Western blots where we compared the intensity of bands due to endogeneous hMLH3 and hPMS2 proteins in HeLa cells with that of bands due to known amounts of the corresponding recombinant proteins (Fig. 2*A*). These experiments revealed that hMLH3 is ~60 times less abundant than hPMS2. Considering that hPMS1 is ~10 times less abundant than hPMS2 in human cells (5), hMLH3 exists in the cells at levels significantly

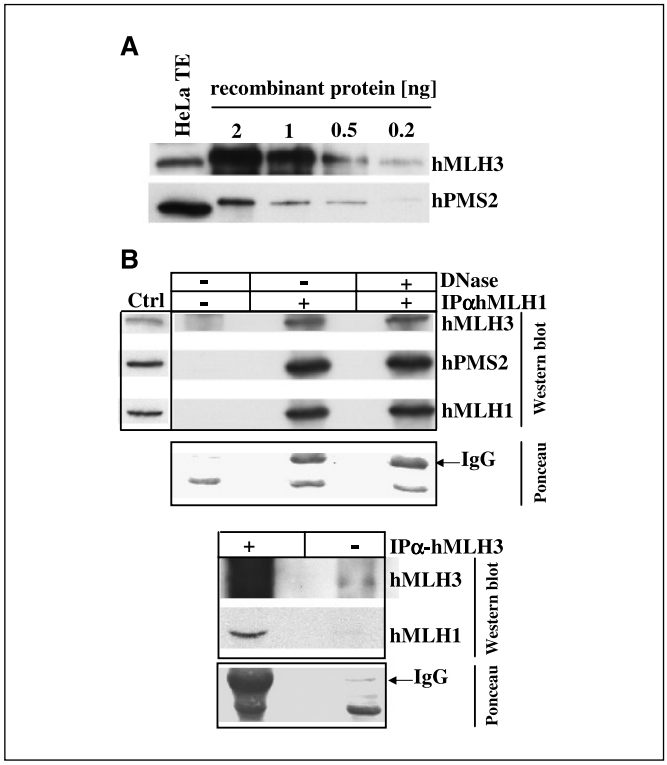

Figure 2. Relative abundance of hMLH3 and its interaction with hMLH1 *in vivo. A,* the recombinant hMLH3 and hPMS2 proteins were loaded onto a denaturing polyacrylamide gel in the indicated amounts and visualized by Western blotting with the respective antibodies. The relative abundance of the two polypeptides was calculated by comparing the intensity of the hMLH3 and hPMS2 bands with those of the endogenous proteins present in 50 μg of HeLa whole cell extract. The blot is representative of two independent experiments, and the intensities of the bands were calculated by densitometry. *B,* coimmunoprecipitation of hMLH3 and hMLH1 in HeLa cells. One milligram of whole cell extract was incubated with or without 6 μg of anti-hMLH1 antibody (*top*) or 10 μg of anti-hMLH3 antibody (*bottom*). *DNase,* reaction done in the presence of 25 units of Benzonase. Ponceau staining for IgG is also shown to show equal loading. *Ctrl,* 50 μg of HeLa whole cell extract.

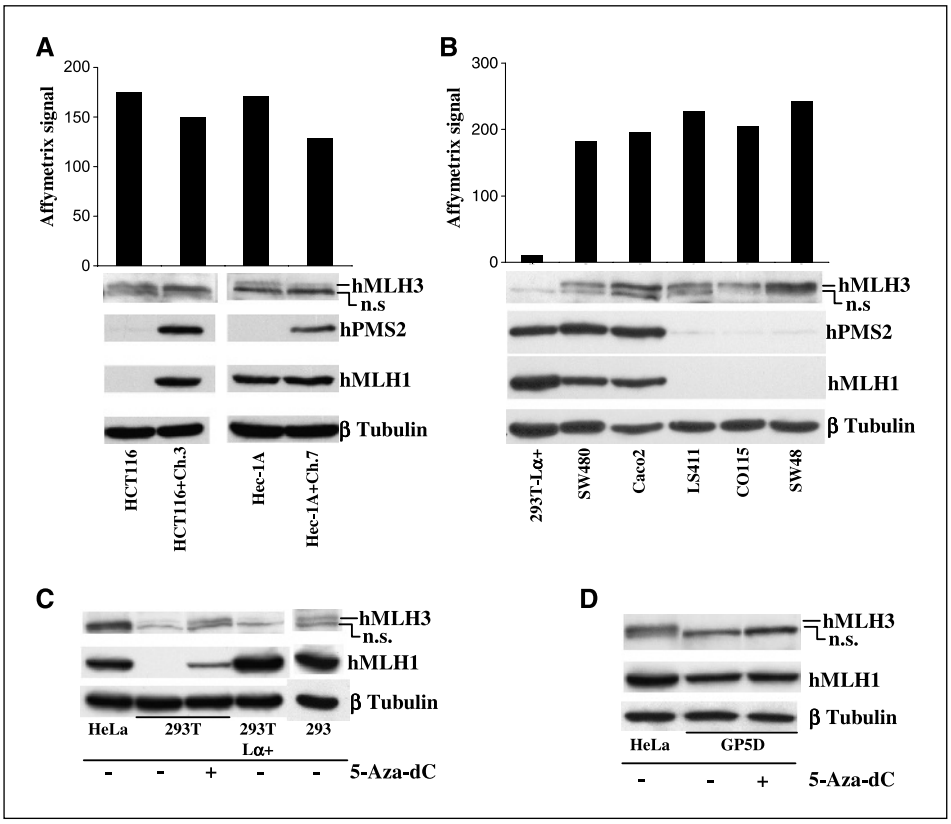

Figure 3. Expression of hMLH3 *in vivo* is independent of hMLH1 and hPMS2 and can be controlled by cytosine methylation. *A,* microarray analysis of *hMLH3* mRNA (*top*) and protein (*bottom*) expression in hMLH1-deficient HCT116 and hPMS2-deficient Hec-1A cells. Correction of the MMR defect by transfer of chromosome 3 or 7, which carry wild-type copies of the *hMLH1* and *hPMS2* genes, respectively, had no effect on hMLH3 expression. *B,* hMLH3 expression is independent of hMLH1/hPMS2 expression in a panel of MMR-proficient and MMR-deficient cell lines. Legend as in (*A*). *C,* the *hMLH3* promoter in 293T cells in silenced by methylation. Expression of *hMLH1* in 293T-Lα cells does not alter hMLH3 levels but demethylation of the promoter by 5-aza-2′-deoxycytidine (*5-Aza-dC*) treatment results in the reappearance of hMLH3, together with hMLH1, the promoter of which is also methylated in these cells. *D,* down-regulation of the *hMLH3* gene in GP5D cells is not mediated by cytosine methylation. The promoter was shown by methylation-specific PCR to be unmethylated and 5-Aza-dC did not reactivate hMLH3 expression in these cells. In the Western blot experiment, 50 μg of total extract were used per lane.

lower than those of the other two hMLH1-interacting partners hPMS2 and hPMS1. In spite of this difference, hMLH3 was found to physically interact with hMLH1 in Far Western experiments (7) and in mammalian two hybrid assays (30). We could confirm this interaction using immunoprecipitation experiments in which the anti-hMLH1 antibody could immunoprecipitate both hMLH3 and hPMS2 from human cell lysates (Fig. 2*B, top*) and the anti-hMLH3 antibody precipitated the endogenous hMLH1 (Fig. 2*B, bottom*). No proteins were detected in control experiments where the precipitating antibody was omitted. The interaction between hMLH3 and hMLH1 was not mediated by bound DNA because treatment with DNase before incubation with the antibodies failed to abolish the interaction between the two proteins (Fig. 2*B, top*).

hMLH1 is not required for the stability of hMLH3 in human cells. hPMS2 and hPMS1 are stabilized by the presence of hMLH1 (1, 31). Considering this characteristic of these two MutL homologues, together with the finding that hMLH1 was required for the stabilization of hMLH3 in baculovirus-infected Sf9 cells (Fig. 1*A*), we expected to observe substantially decreased levels of endogenous hMLH3 in human cell lines lacking hMLH1. Surprisingly, we could detect hMLH3 in hMLH1-deficient HCT116 cells, and the restoration of hMLH1 expression by chromosome 3 transfer resulted in no appreciable increase in hMLH3 level (Fig. 3*A, bottom left*). This shows that the presence of hMLH1 is not required for hMLH3 stability in human cells. The relative amounts of intracellular hMLH3 were also unaffected by hPMS2 levels, as shown by comparison of hMLH3 band intensity in Western blots of extracts of the hPMS2-deficient Hec-1A cells with those of a Hec-1A clone in which the expression of hPMS2 was restored by chromosome 7 transfer (Fig. 3*A, bottom right*). hMLH3 protein levels failed to correlate with hMLH1 and hPMS2 expression also in other colon cancer cell lines, such as SW480 or Caco2, that express both hMLH1 and hPMS2, or LS411, CO115, or SW48 that lack hMutLα (Fig. 3*B*).

Having established that the level of hMLH3 in cells is not dependent on hMLH1 but that it correlates well with *hMLH3* mRNA levels (Fig. 3*B*), we wondered whether the fluctuation of hMLH3 expression in the tested cell lines could be linked with cytosine methylation, which is known to silence several key genes in colon cancer (32). The human embryonic kidney cell line 293T is deficient in both hMLH1 and hMLH3 (Fig. 3*C*) and it could be shown that the CpG islands that constitute the promoters of hMLH1 (25) and other genes (33) are silenced by hypermethylation in these cells. As the *hMLH3* promoter also contains a CpG island, we reasoned that the lack of *hMLH3* expression in this cell line might also be linked to the transcriptional inactivation of its promoter. This prediction was substantiated in two independent experiments. First, treatment of 293T cells with the demethylating agent 5-aza-2′-deoxycytidine partially restored the expression of both hMLH1 and hMLH3 (Fig. 3*C*). In the second experiment, we treated genomic DNA of 293T and the parental 293 cells (which express both hMLH1 and hMLH3; Fig. 3*C*) with sodium bisulfite, which deaminates cytosines to uracils, but leaves 5-methycytosines unchanged. Methylation-specific PCR showed that the promoter of the *hMLH3* gene in 293T cells was indeed methylated (data not shown). As expected, expression of high amounts of hMLH1 in the 293T-derived 293T Lα+ cells resulted in the stabilization of hPMS2 (26) but did not affect hMLH3 levels (Fig. 3*C*). The promoter of the *hMLH3* gene can thus be silenced by cytosine methylation, but this is most likely not the only mechanism that results in the lack of expression of the protein, as 5-aza-2′-deoxycytidine treatment failed to induce the expression of hMLH3 in GP5D cells (Fig. 3*D*).

Role of hMutLγ in *in vitro* mismatch repair. The observation that extracts from 293T-Lα+ cells are MMR proficient (26) despite their lack of hMLH3 suggested that hMutLγ does not play a major role in MMR *in vitro*. However, the possibility that it acts as a backup to hMutLα in the absence of hPMS2 could not be excluded. Therefore, we tested extracts of the human cell line HeLa clone 12, which expresses hMLH1, hPMS1, and hMLH3 but lacks hPMS2. As shown in Fig. 4*A*, these extracts were deficient in the repair of heteroduplex substrates containing either a G/T mismatch or an insertion/deletion loop of one or two nucleotides, but their repair proficiency on all tested substrates could be restored by the addition of recombinant hMutLα. Before concluding that hMutLγ does not participate in MMR, we considered the possibility that the expression level of endogenous hMLH3 in the tested human cell lines might be too low to be detectably active in our *in vitro* assay. Therefore, we decided to test whether *in vitro* MMR activity may be detected in the presence of higher amounts of the heterodimer. These experiments were done with the hMutL α, β, and γ deficient extracts of 293T cells supplemented with whole cell extracts from Sf9 cells expressing comparable amounts of hMutLα or hMutLγ (Fig. 4*B, inset*). As shown previously, extracts of Sf9 cells overexpressing hMutLα could complement the MMR defect in the 293T extracts very efficiently, whereas extracts of uninfected Sf9 cells failed to do so (Fig. 4*B*; ref. 26). Interestingly, when extracts of Sf9 cells expressing hMutLγ were used, we observed an increase in repair activity of ~20%. Similar results were obtained when the hMutLγ was enriched by Ni-agarose chromatography, showing that the observed MMR activity was specific to hMutLγ. We detected similar repair activities on substrates containing a G/T mismatch or a 1-base loop with a nick located either 5′ or 3′ from the mismatch, but no activity was observed on a substrate containing insertion/deletion loops of two or four nucleotides (Fig. 4*B*; data not shown). These experiments show that although physiologic levels of hMutLγ are insufficient to mediate mismatch correction in our *in vitro* MMR assays, the factor can participate, albeit with low efficiency, in the correction of base-base mispairs and one-nucleotide insertion/deletion loops.

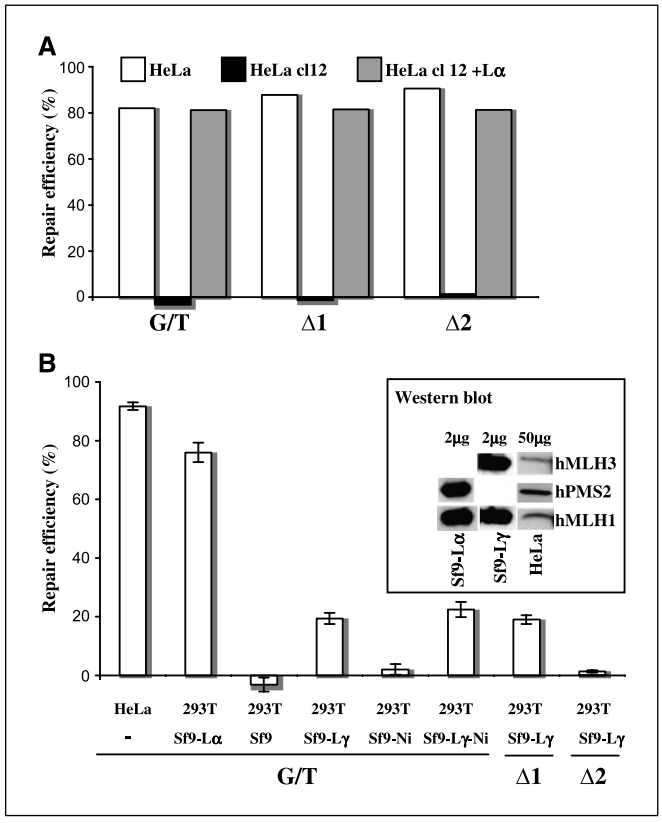

Figure 4. *In vitro* MMR assays. *A,* the MMR defect of cytoplasmic extracts of the hPMS2-deficient cell line HeLa clone 12 can be corrected by the addition of 0.2 μg purified hMutLα. The repair efficiencies were determined on heteroduplex substrates containing a G/T mismatch, or +1 (*Δ1*) or +2 (*Δ2*) insertion/deletion loops. *B,* cytoplasmic extracts of 293T cells, which lack hMLH1, hMLH3, and hPMS2 were supplemented with 2 μg of whole cell extract from Sf9 cells coinfected with hMLH1/hPMS2 (*Sf9-Lα*), hMLH1/hMLH3 (*Sf9-Lγ*), or the latter partially purified on Ni-agarose (*Sf9-Lγ-Ni*). MMR efficiency was tested on heteroduplex substrates containing a G/T mismatch, or +1 (Δ1) or +2 (Δ2) insertion/deletion loops. The amounts of hMutLα and hMutLγ complexes used for complementation were comparable (*inset*). Whole cell extracts from uninfected Sf9 cells (*Sf9*) or Sf9 extract that underwent Ni-agarose (*Sf9-Ni*) chromatography were used as negative controls. *Columns,* result of at least three independent experiments; *bars,* SE. Cytoplasmic extract of the MMR-proficient HeLa cells was used as the positive control.

Discussion

Like its *S. cerevisiae* homologue (8), the mammalian *MLH3* gene (7) could be shown to be involved in meiotic recombination (13–15). However, whereas the *S. cerevisiae* (8) and *Schizosaccharomyces pombe* (12) proteins play a small but distinct role in the repair of a subset of insertion/deletion loops, no similar evidence existed for mammalian MLH3. In this present study, we set out to search for this evidence.

We first wanted to study the expression of hMLH3 and confirm the existence of hMutLγ *in vivo*. Using a newly generated antibody, we showed that hMLH3 is much less abundant than the other two known hMLH1 interactors, hPMS2 and hPMS1. Despite this, we could confirm the physical interaction between hMLH3 and hMLH1 in HeLa cells by immunoprecipitation experiments. Surprisingly, although hMLH1 was required for hMLH3 stability in Sf9 cells (Fig. 1*A*), no such requirement was apparent in human cells where no degradation of hMLH3 occurred in the absence of hMLH1 (Fig. 3*A* and *B*). We also failed to observe any significant competition between hMLH3 and hPMS2 for hMLH1, showing that in human cells hMLH3 might be stabilized by interaction with another, as yet unidentified, protein. This finding is supported by evidence from meiosis in mice, where Mlh3 was seen to bind to pachytene chromosomes before Mlh1 and, after Mlh1 recruitment to these sites, foci containing Mlh3 alone persisted (11, 15). It was, therefore, suggested that Mlh3 could either exist alone or interact with a different partner (11). Immunoprecipitation experiments revealed a direct interaction of *scMlh3p* with *Sgs1* helicase in meiotic *S. cerevisiae* cells (34) and hMLH3 was shown to bind hMSH4 in meiotic human cells (14); however, the identification of the putative hMLH3 partners that might help stabilize it in mitotic cells must await the results of future experiments.

The ultimate objective of this work was to elucidate the role of hMLH3 in human MMR. We first tested extracts of human HeLa clone 12 cells, which lack hPMS2 (22) and thus contain only hMutLβ and hMutLγ. As the former heterodimer is devoid of MMR activity in our *in vitro* MMR assay (31), any observed repair activity could be ascribed to hMutLγ. The extracts were MMR deficient on all tested substrates (Fig. 4*A*), which suggested that the hMutLγ heterodimer does not participate in MMR. However, as hMLH3 is generally much less abundant in human cells than hPMS2, we

wanted to exclude the possibility that the lack of repair activity is linked to insufficient amounts of hMutLγ. Therefore, we tested the MMR activity of extracts of 293T cells, which are deficient in all three MutL homologues, supplemented either with recombinant hMutLα or hMutLγ (Fig. 4*B*). The former factor complemented the MMR defect in the 293T extracts on all tested substrates. When comparable amounts of hMutLγ were used, we observed a small but significant (~20%) repair with both G/T and +1 insertion/deletion loop substrates. This repair activity was not due to an intrinsic repair activity of the Sf9 extracts per se, as extracts from uninfected Sf9 cells were repair deficient in the complementation experiments. As there are no available functional assays to test the activity of hMutLγ, a possibility exists that this heterodimer was isolated in a partially inactive form. However, we consider this possibility unlikely because all the procedures used were identical to those used for the preparation of the Sf9 extract expressing hMutLα, which was fully active. Moreover, immunoprecipitation experiments done with Sf9 extracts expressing hMutLγ showed that hMLH3 was able to bind hMLH1 (data not shown). The sensitivity of the *in vitro* MMR assay remains, however, rather low so that the contribution of hMutLγ to the repair process *in vivo* might be higher. Interestingly, the repair activity of hMutLγ was limited to G/T mismatch and 1-base loops, as we failed to observe any repair activity using +2- and +4-base-loop substrates. The latter result indicates that hMutLγ seems to be involved in the repair of substrates recognized by hMutSα rather than insertion/deletion loops of more than one extrahelical nucleotide recognized by hMutSβ. This is in contrast to the data obtained in *S. cerevisiae* where the role of *scMlh3p* seems to be in the repair of a subset of insertion/deletion loops together with *scMutSβ*. The role of hMLH3 in mammals thus might differ from that in lower eukaryotes.

Our findings, suggesting that hMutLγ may play a backup role in human MMR, are supported by evidence from the mouse model. As noted above, Mlh3 *null* mice were not cancer prone in the first 9 months of life and showed no gross defects in MMR (15). However, a long-term study of these animals, coupled with a highly sensitive analysis of their genomic DNA, provides evidence for the involvement of Mlh3 defects in both MMR and tumorigenesis. $Mlh3^{-/-}$ mice have a shorter life span than the wild-type controls and more than half of the animals develop cancers, including gastrointestinal tumors after the 9-month time span. Importantly, Mlh3 deficiency increased the levels of mutations in long mononucleotide repeats, although to a lesser extent than in $Pms2^{-/-}$ mice (35). Taken together, our results and the mouse model data suggest that the hMutLγ heterodimer functions in the repair of base-base mismatches and small insertion/deletion loops.

Considering the possible involvement of hMLH3 in human MMR, the identification of hMLH3 silencing through promoter hypermethylation is of particular interest. We showed that the hMLH3 promoter is methylated in 293T cells and that the protein is consequently not expressed. In this particular cell line, the methylation could be caused by the presence of the SV40 large T antigen. However, using methylation-specific PCR, we could detect partially methylated hMLH3 promoters in the colon cancer cell line LS411 and in the ovarian cancer cell line A2780/CP70, and fully methylated in the leukemia cell line Jurkat (data not shown), which shows that hMLH3 silencing via promoter hypermethylation can also be unrelated to the presence of SV40 large T antigen.

Although recombinant hMutLγ possessed detectable repair activity in our *in vitro* MMR assays, hPMS2-deficient cells expressing hMLH3 display a strong mutator phenotype (refs. 16, 23; this study). This suggests that hMLH3, most likely in the form of hMutLγ, does not play a major role in MMR *in vivo*. However, the detection of sequence variants of hMLH3 in the germ line of families predisposed to colorectal cancer (17, 20), coupled with our detection of epigenetic silencing of hMLH3 in human cell lines, suggests that this gene may play a role in human cancer, possibly in combination with other risk factors. If hMutLγ does indeed play a backup role for hMutLα *in vivo*, the fluctuating abundance of hMLH3, such as that observed in the tested cell lines (Figs. 1 and 3), might help explain the variable penetrance of *hPMS2* mutations in hereditary nonpolyposis colon cancer families (16).

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4.2

Identification of Interacting Partners of hMLH1 and hPMS2 by Tandem Affinity Purification

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ABSTRACT

Postreplicative mismatch repair (MMR) involves the concerted action of at least 20 polypeptides. Although the minimal human MMR system has recently been reconstituted *in vitro*, genetic evidence from different eukaryotic organisms suggests that some steps of the MMR process may be carried out by more than one protein. Moreover, MMR proteins are involved also in other pathways of DNA metabolism, but their role in these processes is unknown. In an attempt to gain novel insights into the function of MMR proteins in human cells, we searched for interacting partners of two key MMR proteins, MLH1 and PMS2, by tandem affinity purification (TAP). Our approach was validated by the finding that most of the known interacting partners of MLH1 and PMS2 were present in our protein mixtures. However, we also identified a large number of other polypeptides, some of which bound to the MMR proteins with very high affinity, as shown in reciprocal immunoprecipitation assays. Whether these polypeptides represent novel members of the mismatch repairosome, or whether they interact with the MMR proteins during other metabolic transactions is currently the subject of intense study in our laboratory.

INTRODUCTION

The evolutionarily conserved human mismatch repair (MMR) system maintains genomic stability by removing replication errors from DNA (Marra G. and Jiricny J., 2005). Despite the fact that the human MMR pathway was recently reconstituted *in vitro* from purified individual components (Dzantiev L. et al., 2004; Zhang Y. et al., 2005), our knowledge of the molecular mechanisms of this process is still incomplete. The repair reaction requires a mismatch recognition step, which is mediated by the heterodimers hMutSα (hMSH2 and

hMSH6) or hMutSβ (hMSH2 and hMSH3). hMutSα preferentially recognizes single base mismatches and insertion-deletion loops (IDLs) of 1-4 bases, whereas hMutSβ recognises IDLs containing 1-8 bases. Upon mismatch binding, the hMutS heterodimer associates with the heterodimeric complex hMutLα (hMLH1 and hPMS2) that was shown to be essential for repair. However, the biochemical function of the MutL proteins remains enigmatic. hMutLα is believed to couple the mismatch recognition step to downstream processes that include the removal of the mismatch from the nascent DNA strand, resynthesis of the degraded region and ligation of the remaining nick (reviewed in Kunkel T.A. and Erie D.A., 2005; Marra G. and Jiricny J., 2005).

hMutLα was shown to possess a weak ATPase activity, which most likely plays a role in signalling rather then in catalysis (Acharya S. et al 2003; Raschle M. et al., 2002). hMLH1 can bind two other human MutL homologues, hPMS1 and hMLH3 to form the heterodimers hMutLβ and hMutLγ, respectively. *In vitro* studies failed to identify a role of hMutLβ in MMR (Raschle M. et al., 1999), whereas hMutLγ can participate in the repair of base-base mismatches and small IDLs, even though its *in vivo* role seems to be only marginal (Cannavo E. et al., 2005). A better understanding of the mechanism of human MMR and, in particular, of the function of hMLH1 is of particular importance due to the link between MMR and cancer. Mutations in *MMR* genes predispose to hereditary non polyposis colorectal cancer (HNPCC), with *hMLH1* mutations being responsible for most (≈60%) of the cases (HNPCC mutation database at http://www.insight-group.org/).

In addition to being involved in MMR, hMLH1 was shown to have a role in meiotic recombination; $Mlh1^{-/-}$ knock out mice develop a spectrum of tumours that resemble the tumour spectrum of the $Msh2^{-/-}$ mice, but the $Mlh1^{-/-}$ mice are, in addition, sterile. Sterility is also a feature that characterizes $Pms2^{-/-}$ male mice, suggesting that Mlh1 and Pms2 have different meiotic functions (reviewed in Wei K et al 2002; Jiricny J. and Marra G., 2003).

In conclusion, in spite of the enormous progress in the characterization of the human MMR machinery during the last decade, many unanswered questions remain. Among those, understanding the biochemical and biological role of hMutLα, hMutLβ and hMutLγ is of particular importance, as well as the identification of additional factors that might play a direct or regulatory role in the repair process. Nothing is known, for instance, about the regulation of the MMR machinery or the potential participation of DNA helicases in the repair process. In addition, the *in vivo* repair mechanism might be characterized by functional redundancies, such as in *E. coli*, where four different exonucleases are involved (reviewed in Schofield M.J. and Hsieh P., 2003). Reconstitution of MMR *in vitro* thus does not exclude the involvement of other, yet unidentified, factors in the repair process.

In an attempt to clarify the function of hMutLα, we searched for new interacting partners of hMLH1 and hPMS2 using Tandem Affinity Purification (TAP). Several reports provide evidence that this technique, originally tested in *S. cerevisae* (Rigaut G.et al 1999; Puig O. et al 2001), represents a major improvement in the identification of protein-protein interactions. TAP represents a valuable method to identify interacting proteins *in vivo,* under native conditions and with a high degree of purity (Gingras A.C. et al., 2005). Our analysis resulted in the identification of a number of proteins in complex with hMLH1 or hPMS2. While some of these interactors were described previously, a majority of these proteins represent novel, either directly or indirectly interacting, partners. We started to analyse and validate some of these potentially interesting interactions and will focus on their role in human MMR or related pathways.

METHODS

Plasmid construction

The mammalian vector for the expression of N-terminally TAP-tagged hMLH1 was created by inserting the cDNA encoding the full-length hMLH1 into the *Eco*RI site of pZome-1-N (Cellzome), and the vector for the expression of C-terminally TAP-tagged hPMS2 was created by inserting the cDNA encoding the full-length hPMS2 into the *Bam*HI site of pZome-1-C (Cellzome).

Cell culture and transfection

The 293T and HeLa cells were obtained from the cell line repository of Cancer Network Zurich and the HeLa12 cell line was kindly provided by Dr. M. Bignami (ISS, Rome, Italy). All the cell lines were cultured at 37°C in a 5% CO_2-humidified atmosphere and maintained in the appropriate media. Transfection was performed using the Fugene 6 transfection reagent (Roche Applied Science) according to the manufacturer's recommendations. For generation of stable cell lines, 0.2 μg/ml of Puromycin (Invivogen) was added to the medium one day after transfection. After 2 weeks the surviving colonies were isolated and their extracts were screened by Western blotting using antibodies against hMLH1 and hPMS2. The clones showing the highest expression of the two tagged mismatch repair proteins were further subcloned.

Western blot analysis and antibodies

Western blot analysis was performed as described previously (Cejka P. et al., 2003) using the following antibodies: anti-hMLH1 and -hPMS2 from BD Pahrmingen (1:4000 and 1:1000, respectively) and anti β-Tubulin from Santa Cruz, (1:2000). For the immunoprecipitation experiment, the anti-hPMS1 rabbit polyclonal antibody (Raschle M.

et al., 1999) was further affinity-purified. Briefly, 10 mg of purified $(His)_6$-tagged internal peptide of hPMS1 (aa 335-64; Raschle M. et al., 1999) were coupled to 0.4 gr of CNBr-activated Sepharose 4B (Amersham Pharmacia), according to the manufacturer's instructions. 5 ml of rabbit polyclonal anti-hPMS1 serum diluted 10x in 50 mM Tris-HCl pH 7.5 was then bound to the CNBr-bound antigen for 4 h at 4°C. After two washes in 10 mM Tris-HCl pH 7.5 and two additional washes in 10 mM Tris-HCl pH 7.5, 500 mM NaCl, the antibody was eluted with 100 mM Glycine-HCl pH 2.5 at 4°C. The elution step was repeated twice and the final eluates were pooled in new tubes containing Tris-HCl 1 M, pH 8.0 to a final concentration of 100 mM. 300 μl of the corresponding pre-immune serum were IgG/A purified by binding to 300 μl of Protein A/G Plus agarose (Santa Cruz Biotechnology). Elution of the IgG/A bound antibodies was then performed as above.

Co-immunoprecipitation

Co-immunoprecipitation analyses were performed as described previously (Cannavo E. et al., 2005) using 1 mg of whole cell extracts and 6 μg of anti-hMLH1 antibody (BD Pharmingen). Control experiments were done in the absence of the primary antibody. Large-scale immunoprecipitation of hPMS1 was performed using 10 mg of HeLa whole cell extract and 1.6 μg of affinity-purified rabbit polyclonal anti-hPMS1 or purified pre-immune serum as negative control.

Testing of MMR status

In vitro MMR assay, MNNG sensitivity assay and FACS analysis were performed as described previously (Cejka P. et al., 2003)

Tandem Affinity Purification (TAP)

293T and HeLa12 cells stably transfected with plasmids expressing the N-terminally TAP-tagged hMLH1 and the C-terminally TAP-tagged hPMS2 (TAP-hMLH1/293T and TAP-hPMS2/HeLa12 cell lines respectively) were plated in 15 cm dishes. Cells were cultured to 80% confluency, washed twice in cold PBS and lysed 30 min on ice in 50 mM Tris-HCl pH 8.0, 125 mM NaCl, 1% NP40, 2 mM EDTA, 1 mM phenylmethylsulfonylfluoride (PMSF), 1x complete inhibitory cocktail (Roche Molecular Biology), 0.5 mM sodium orthovanadate, 20 mM sodium fluoride and 5 nM okadaic acid. The lysates were cleared by centrifugation at 12000 x*g* for 3 min and the soluble material was collected. Protein concentration was determined using the Bradford assay (Bio-Rad).

Batch Tandem Affinity Purification was performed according to the original protocol (Puig O. et al., 2001) with minor changes. All the following purification steps were performed on ice or at 4°C. For each experiment, 60 mg of whole cell extract was incubated for 4 h with gentle agitation with 100 µl of IgG Sepharose beads (Amersham Biosciences) equilibrated with lysis buffer. Beads were then washed 3x with 1 ml lysis buffer and 3x with 1 ml TEV buffer (10 mM Hepes-KOH pH 8.0, 150 mM NaCl, 0.1% NP40, 0.5 mM EDTA, 1 mM DTT, 1mM PMSF and 1x complete inhibitory cocktail). Bound TAP-tagged proteins were released by overnight incubation in TEV buffer containing 16 U of acTEV protease (Invitrogen) in tubes mounted on a rotating platform. The supernatant from the TEV reaction was collected and transferred to a new tube. 1 volume of Calmodulin Binding Buffer (CBB: 10 mM β-mercaptoethanol, 10 mM Hepes-KOH pH 8.0, 150 mM NaCl, 1 mM MgOAc, 1 mM imidazole, 0.1% NP40, 2 mM $CaCl_2$, 1 mM PMSF and 1x complete inhibitory cocktail) was added to the collected supernatant and centrifuged at 1500 rpm for 3 min. The supernatant was then transferred to a new tube and the procedure described above was repeated two more times. 1/250 volume of 1 M $CaCl_2$ was then added and the

supernatant was batch-purified by binding to 100 µl of calmodulin affinity resin (Stratagene) equilibrated in CBB, for 4 h on a rotating platform. Beads were washed 3x with 1.2 ml of CBB and 2X with 1.2 ml of Calmodulin Rinsing Buffer (CRB: 50 mM ammonium bicarbonate pH 8.0, 75 mM NaCl, 1 mM MgOAc, 1 mM imidazole and 2 mM $CaCl_2$) and eluted with 100 µl of Calmodulin Elution Buffer (CEB: 50 mM ammonium bicarbonate pH 8.0 and 35 mM EGTA). One third of the eluate was separated by SDS-PAGE and visualized by silver staining. As negative control, the purification was performed with extracts prepared from parental cells not expressing the tagged protein.

Mass Spectrometry (MS)

The eluate from two TAP experiments (total volume 200 µl) was concentrated using the Microcon YM-3 concentrator (Millipore) according to the manufacturer's instructions, separated by 7.5% SDS-PAGE and visualized by Coomassie staining. The gel was then cut into 11 pieces and subjected to in-gel tryptic digestion. Briefly, the gel pieces were additionally cut into smaller fragments and subjected to two cycles of rehydration in 50 mM ammonium bicarbonate and shrinking by dehydration in 80% acetonitrile. Proteins were then reduced with 37 mM DTT in 50 mM ammonium bicarbonate at 50°C, 30 min. After two rounds of dehydration in 80% acetonitrile, proteins were alkylated with 20 mM iodoacetamide in 50 mM ammonium bicarbonate, 15 min at room temperature in the dark. After 3 rounds of rehydration in 50 mM ammonium bicarbonate and shrinking in 80% acetonitrile the gel pieces were incubated with 200 ng of sequencing grade modified trypsin (Promega) for 4 hours at 37°C and then 25°C overnight. Peptides were extracted by one change of formic acid 0.1% and three changes of 80% acetonitrile and dried under vacuum.
The hPMS1- or pre-immune serum-bound proteins from the large scale immunoprecipitation (see above) were analyzed by SDS-PAGE, stained with Coomassie blue, the gel was cut into 15 pieces and subjected to tryptic digestion according to the same protocol.

Tryptic peptides were analyzed on a LTQ FT™ (Thermo Electron, Bremen, Germany). Peptides were separated on a nano-HPLC (Agilent, Palo Alto, CA) online prior to MS analysis on a C18 reversed phase column using an acetonitrile/water system at a flow rate of 200 nl/min. Tandem mass spectra were acquired in a data dependent manner. Typically 4 MS/MS were performed after each high accuracy survey scan. The human portion (taxonomy ID: 9606) of the UniProt database (http://www.uniprot.org) was interrogated using the Mascot search algorithm (Perkins D.N. et al., 1999).

RESULTS

Generation and characterization of stable mammalian cell lines expressing TAP-tagged hMLH1 and hPMS2

Tandem Affinity Purification (TAP) was shown to be a powerful method for identification of interacting partners of known proteins in various host cell lines (Puig O. et al., 2001; Gingras A.C. et al., 2005). In order to avoid competition, it is preferable to use cell lines lacking the corresponding endogenous protein. For this reason, we stably-transfected two human cell lines deficient for hMLH1 or hPMS2, namely the hMLH1-deficient embryonic kidney cell line 293T and the hPMS2-deficient ovarian carcinoma cell line HeLa12 with pZome-1-N-hMLH1 and pZome-1-C-hPMS2, respectively. The resulting clones were analyzed by Western blot for the expression of the TAP-tagged protein hMLH1 or hPMS2 (see *Methods* for details). The clones exhibited various expression levels of the transfected proteins (data not shown). The two selected stable cell clones expressed the TAP-tagged protein at comparable levels to the amount present in the MMR-proficient cell line HeLa (Fig 1A). The TAP-tagged protein in both cases translocated into the nucleus as ascertained by immunofluorescence or immunoistochemistry (data not shown).

To rule out the possibility that the TAP-tag impairs the function of hMLH1 or hPMS2, we performed *in vitro* MMR assay with cytoplasmic extracts from TAP-hMLH1/293T and TAP-hPMS2/HeLa12 cells. As shown in Fig.1B, the MMR activity in both cell lines was

comparable to the repair activity of MMR-proficient HeLa cells. Treatment of mammalian cells with low doses of S_N1 type alkylating agents, such as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), induces a G_2 cell cycle arrest that is absolutely dependent on functional MMR. Thus, loss of MMR leads to increased resistance to killing by these drugs. We confirmed this hallmark of MMR proficient cells in our stable cell lines by FACS analysis. Both TAP-hMLH1/293T and TAP-hPMS2/HeLa 12 cell lines arrested in the G_2 phase of the cell cycle upon treatment with 0.2 μM of MNNG for 24 h (Fig. 1C). The arrest of cell growth upon treatment with MNNG was also confirmed by clonogenic assays (data not shown). In summary, we show that the TAP-tag does not impair the MMR function of hMLH1 or hPMS2, respectively.

Tandem Affinity Purifications (TAP)

Since its first description in 1999 (Rigaut G. et al., 1999) TAP has been successfully used in the identification of binding partners of various proteins (Puig O. et al 2001 and reviewed in Gingras A.C. et al., 2005). The TAP-tag consists of two IgG binding domains of the *S. aureus* protein A and a calmodulin binding peptide separated by a TEV protease cleavage site. The interaction of hMLH1 and hPMS2 is mediated *via* the C-terminus of the hMLH1 and the N-terminus of hPMS2 (Kondo E. et al., 2001). To avoid a possible impairment of the reciprocal binding, we placed the TAP-tag at the N-terminus of hMLH1 and at the C-terminus of hPMS2 (Fig.2A). The protein complexes were then isolated by chromatography on IgG-Sepharose, followed by elution with TEV protease, and loading into a calmodulin Sepharose column. The final elution was carried out with EGTA (Fig. 2B). TAP was performed as described in *Materials and Methods*. Each experiment was repeated several times and the results were highly reproducible, as judged by comparing silver stained gels from independent experiments (data not shown).

Identification of interacting partners of hMLH1 and hPMS2

For each large-scale experiment, TAP was performed with 120 mg of whole cell extract and, upon concentration, the final eluate was subjected to electrophoresis on a polyacrylamide gel

and visualized by Coomasie staining. The lane containing the sample was then cut into 11 pieces, and the identity of the proteins was established by MS analysis. In both cases, the bait protein and its principle interacting partners were detected as the most prominent bands, migrating at the predicted molecular size (Fig. 3A). The identity of these proteins was verified not only by MS analysis, where the corresponding Mascot score was always among the highest (Table 1 and 2), but also by Western blot analysis (Fig. 3B), therefore validating this approach for the identification of physiological binding partners. We could identify a number of proteins specifically present in the eluate from TAP-hMLH1 or TAP-hPMS2. First, we verified that the detected proteins were isolated from the gel area corresponding to the predicted molecular size. Next, we classified the proteins into several groups according to the known function. Selected hMLH1 interactors are shown in Table 1, and selected hPMS2 interactors in Table 2.

Co-immunoprecipitation of hPMS1 interacting partners.

hPMS1 interacts *in vivo* with hMLH1 to form the heterodimer hMutLβ. hMutLβ was shown not to have a role in *in vitro* MMR (Raschle M. et al., 1999) and the physiological role of hPMS1 has yet to be identified. As expected, we found hPMS1 associated with hMLH1 in the TAP eluate from the cell line expressing TAP-tagged hMLH1 and not TAP-tagged hPMS2 (Table 1 and 2). It was indeed shown previously that hPMS2 and hPMS1 compete for the same interacting region of hMLH1 (Kondo E. et al., 2001). The relative abundance of hPMS1 found in complex with hMLH1 was instead a surprising finding. As shown in Fig. 4A, the band corresponding to hPMS1 is one of the three major bands, the other two being the bait protein hMLH1 and its major interactor hPMS2. In Western blot analysis, the relative amount of hPMS1 present in the hMLH1-TAP eluate appeared comparable to the amount of hPMS2 in the whole cell extract (Fig. 4B). Given that the level of hPMS1 was reported to be lower than that of hPMS2 (Raschle M. et al., 1999), the fact that they were pulled down in similar amounts shows that the affinity of hMLH1 for hPMS1 is high. Considering the strength of the interaction and the relative amount of hPMS1 in the TAP eluate, it appears unlikely that hMutLβ has no biological role. We thus set out to identify additional interacting

partners of hPMS1, in the hope that they might point at the biological function of hMutLβ. Unfortunately, as no human cell lines lacking hPMS1 have been identified to date, we could not use the TAP approach as the tagged polypeptide might compete with the untagged protein in the cell. We were thus forced to use a large-scale co-immunoprecipitation coupled with MS analysis.

We immunoprecipitated hPMS1 from 10 mg of HeLa whole cell extract, using an affinity-purified anti-hPMS1 antibody or, as a negative control, the purified pre-immune serum. The immunoprecipitates were analyzed by SDS-PAGE, the bands visualized by Coomassie staining (Fig. 5A), the lanes containing the sample were cut into 15 pieces, which were subjected to in-gel tryptic digestion and the peptides were analyzed by MS as described. The affinity-purified anti-hPMS1 antibody efficiently immunoprecipitated hPMS1 and its major partner hMLH1 from whole cell extracts while the pre-immune serum failed to do so (Fig. 5B). We could identify a high number of additional specific interacting partners, a sub-set of which was divided into functional categories, and is listed in Table 3.

DISCUSSION

Human MMR has recently been the focus of intensive research efforts, however the precise function of the hMutlα heterodimer remains unclear. In order to elucidate the role of hMLH1 and hPMS2 in MMR, we searched for interacting partners of the two proteins, using the dual-purification strategy TAP which has proven to be an invaluable method for the characterization of protein complexes. This technique was first tested in *S. cerevisiae* (Rigaut G. et al., 1999), but has recently been successfully used in other organisms, including human cells. Several studies compared TAP with single-tag purification strategies, and/or immunoprecipitation experiments. TAP was shown to be significantly more specific, thereby dramatically reducing false positive identifications (reviewed in Gingras A.C. et al., 2005). In *S. cerevisae,* where large datasets are already available, the error rate of the TAP-tag method has been estimated at about 15%, while for a single-epitope tag method the error rate was about 50% (Dziembowski A. and Seraphin B., 2004). In addition, TAP uses mild washing conditions, thus allowing for the recovery of native complexes. When performing TAP the

expression level of the tagged protein is an important determinant for the success of the experiment. For this reason, is it preferable to avoid the use of extracts from transiently-transfected cells, where the expression levels of the tagged proteins are often high, resulting in the identification of a large number of unspecific interactors binding to the overexpressed or misfolded protein and making the identification of low abundant binding partners more difficult. In contrast, performing stable transfections allows selection of clones expressing the recombinant protein at levels comparable to wild type. Moreover, endogenous proteins might compete for binding partners with the stably-expressed TAP-tagged protein and so reduce the recovery of interactors. To avoid this problem, it is preferable to stably-transfect a cell line originally deficient for the target protein. This approach also enables testing the activity of the tagged protein in cell extracts, providing that an appropriate assay is available. In this study, we used hMLH1-deficient 293T cells (Trojan J. et al., 2002) for transfection with TAP-tagged hMLH1 and hPMS2-deficient HeLa12 cells (Ciotta C. et al., 1998) for transfection with TAP-tagged hPMS2, generating two stable cell lines, TAP-hMLH1/293T and TAP-hPMS2/HeLa12, respectively. The interaction domain between the two molecules is located at the C-terminus of hMLH1 and at the N-terminus of hPMS2 (Kondo E. et al., 2001). To avoid a disturbance in the reciprocal interaction, we located the TAP-tags at the N-terminus of hMLH1 and at the C-terminus of hPMS2. TAP was then performed using whole cell extracts from the newly-generated cell lines and, as negative control, from the parental untransfected cells.

A high number of proteins in complex with both TAP-hMLH1 and TAP-hPMS2 were identified by MS analysis. We first validated the approach by demonstrating that hMLH1 pulled down high amounts of its principal interacting partner hPMS2 and *vice versa*. Both proteins were detected by MS with a very high score and were clearly visible on silver-stained SDS gel. Moreover, we could identify hPMS1, a well-known interacting partner of hMLH1 in our hMLH1-TAP pulldown. The interaction of hMLH1 with hMLH3, which form the third hMLH1-containing heterodimer, could not be confirmed, due to the lack of expression of hMLH3 in the 293T cell line because of promoter hypermethylation (Cannavo E. et al.,

2005). Our analysis revealed several additional previously known interactors. Among those, the 5'-3' exonuclease EXO1, which was shown to interact with hMLH1/hPMS2 in co-immunoprecipitation and pulldown experiments (Schmutte C. et al.,2001; Nielsen F.C. et al., 2004), was specifically present also in both our hMLH1- and hPMS2-TAP pulldowns. The EXO1-binding proliferating cell nuclear antigen (PCNA) was shown to interact with hMLH1 in yeast two-hybrid and co-immunoprecipitation experiments (Umar A.et al., 1996; Gu L. et al., 1998). Although we did not identify peptides belonging to PCNA in the eluate from TAP-hMLH1 with a significant score, the protein was specifically present at significant levels in the complex bound to hPMS2. In addition to EXO1 and PCNA, we could detect other proteins involved in MMR, including hMSH2, hMSH6 and replication factor C (RFC), specifically among the hPMS2-bound proteins. Interestingly, all factors required for the recently reconstituted MMR reaction *in vitro* (Dzantiev L. et al., 2004), with the notable exception of DNA polymerase δ and RPA, were specifically detected in our hMLH1- or hPMS2-TAP eluates. hMLH1 was also described to associate with the breast cancer susceptibility gene BRCA1 (Wang Y. et al., 2000) in the so-called BASC complex. The same group later described the binding of BRCA1 to SMC1 (structural maintenance of chromosome protein 1) upon DNA damage (Yazdi P.T. et al., 2002). The finding of both proteins, BRCA1 and SMC1, in our TAP-hMLH1 eluate is a further validation of our experimental conditions. Although several interactions between our bait proteins and known interacting factors could be confirmed with our TAP strategy, this was not always the case. For instance, we failed to detect interactions between hMLH1 and the Bloom helicase (Pedrazzi G. et al., 2001), hMRE11 (Her C. et al., 2001) or MBD4 (MED1) (Bellacosa A. et al., 1999). These interactions were identified using different methods in different cell lines, which might strongly influence protein recovery. In

addition, the strength of the interaction and the location of the TAP-tag at one extremity of the bait protein might have a significant influence on the binding of certain molecules.

Although the interaction between hMLH1 and hPMS1 is very well known, we were puzzled by the amount of the latter protein in our pulldown and consequently by its high affinity to hMLH1. In order to identify interacting partners of this polypeptide, we performed a large-scale immunoprecipitation of hPMS1 in HeLa cells followed by MS analysis. This kind of approach is not comparable to TAP in terms of accuracy and specificity. Antibodies typically cross-react with more proteins than their respective antigens so that the immunoprecipitate contains multiple proteins that are pulled down by the antibody but are not related to the target complex. In the case of hPMS1, the TAP could not be used mainly due to the lack of a hPMS1-deficient cell line as discussed above. In addition, given that the function of hPMS1 is not yet known, it would not be possible to test whether the addition of the TAP-tag impairs the functionality of the protein and so the binding to some, maybe essential, binding partners. Identification of interacting partners by immunoprecipitation is a valid approach,but it requires even more accurate data verification than TAP.

The main focus of this study was to detect novel interactions of proteins with human MutL-homologue complexes, in an attempt to explain the role of these proteins in MMR and related processes. Our study clearly led to the identification of novel interactors (Table 1-3); though it is essential to validate TAP or IP approaches with independent techniques in different cell lines, and ultimately, to eluycidate the biological roles of these interactions. Recently, we started to focus our attention on several potentially interesting molecules identified in our analysis. The presence of numerous proteins belonging to the Ubiquitin pathway in complex with hPMS1 and in particular the very high Mascot score associated to the ubiquitin-ligase EDD1 brought us to investigate the possibility that this molecule

undergos ubiquitylation. Preliminary data show that hPMS1 is indeed poly-ubiquitinated in the cells, in the absence of any treatment (data not shown). The biological significance of this modification, namely whether the poly-ubiquitination merely targets the protein for proteasome-mediated degradation, or whether it modulates hPMS1 activity, remains to be established. The presence of some deubiquitinating enzymes such as UBP5 and UBP5 suggests that poly-ubiquitination is a dynamic process. Interestingly, we could not detect any biochemical activity of hPMS1 expressed in baculovirus infected *Spodoptera frugiperda* cells in our *in vitro* MMR assay (Raschle M. et al., 1999). It will be interesting to evaluate the possibility that ubiquitinated hPMS1 plays a role in postreplicative MMR or in other processes of DNA metabolism.

The newly-identified protein Angiomotin appears to be in a stable complex with hMLH1 (Table 1, score 4013). This protein was identified by a yeast two-hybrid assay in search for Angiostatin interactors. Angiomotin is involved in angiogenesis and is located at the cellular membrane (Troyanovsky B. et al., 2001). Two forms of Angiomotin were identified to date, a shorter form, Angiomotin p80, and a longer form, Angiomotin p130, containing an additional 490aa at the N-terminus, (Bratt A. et al., 2005). We confirmed the interaction between hMLH1 and Angiomotin p130 by immunoprecipitation and could detect Angiomotin in the cytoplasm of 293T cells by immunofluorescence experiments (data not shown). In summary, though hMLH1 is a nuclear protein, it is synthesized in the cytoplasm where it seems to bind Angiomotin. The biological significance of this interaction remains to be established.

Of particular interest appears the identification of the BRCA1-associated C-terminal helicase BRIP1 (BACH1), that was recently identified as FANCJ, in the hMLH1-, hPMS2- and hPMS1-bound complexes (Litman R. et al., 2005; Levran O. et al., 2005; Levitus M. et al., 2005). BRIP1 appears to be critical for homologous recombination and seems to

participate in DNA double strand breaks repair (Cantor S.B. et al., 2001; Litman R. et al., 2005). We confirmed the binding of BRIP1 to hMLH1 by reciprocal immunoprecipitation. BRCA1 was also present in the in the complex (data not shown). It will be important to establish whether BRIP1 plays a role in MMR, or whether it could mediate the link between MMR and recombination. hMLH1 is known to be involved in both mitotic and meiotic recombination as evident from the phenotype of the $Mlh1^{-/-}$ knock out mice that are sterile (Backer SM et al., 1996 and reviewed in Wei K. et al., 2002) and from experiments in yeast cells (Hoffmann E.R. and Borts R.H., 2004).

A number of additional proteins, the peptides of which were identified in our samples, are in our view of particular interest and will be the subject of our future studies. Among those, a complex of two proteins related to the bacterial ATP-dependent helicase RuvB, RuvBL1 (TIP49a) and RuvBL2 (TIP49b), was identified specifically in association with both hMLH1 and hPMS2. RuvBL1 and RuvBL2 are highly conserved in evolution end essential for viability in yeast. The precise role of these ATPase-helicases is not known, but they were reported to be associated with transcription factors (Bauer A. et al., 1998; Cho S.G. et al., 2001), to modulate apoptosis (Dugan K.A. et al., 2002), oncogenic transformation (Wood MA et al., 2000; Feng Y. et al., 2003) and were shown to be in chromatin remodelling complexes in yeast (Shen et al., 2000; Jonsson Z.O. et al., 2004) as well in a complex with the histone acetyltransferase TIP60 in human cells (Ikura T. et al., 2000; Frank S.R. et al., 2003).

It is known that both hMLH1 and hPMS2 contain one monopartite nuclear localization signal (NLS) and that certain mutations within this NLS impair their nuclear import (Wu X et al., 2003; Brieger A. et al., 2005). NLS are recognized by specialized transport factors, karyopherins or importins, which function as heterodimeric protein complexes, that dock NLS-containing substrates and mediate their import into the nucleus (reviewed in Goldfarb

D.S. et al., 2004). We identified importin α2 and its known binding partner importin β1 in the complex with both hMLH1 and hPMS2 (only importin α2 in complex with hPMS1). This finding suggests that importin α2/β1 heterodimer might be the transporter molecule for hMutLα. We also identified several proteins with unknown function. The presence of the protein KIAA1018 appears to be most significant, because of the numerous peptides observed in the complex bound to hMLH1, hPMS2 and hPMS1. KIAA1018 appears to be identical to the myotubularin-related protein 10 (MTMR10). In human, 13 myotubularin-related proteins are known, although the function of many of them has not been identified yet. These proteins are mainly characterized by a tyrosine-phosphatase domain and have been involved in phosphoinositide metabolism, cellular growth and differentiation and found mutated in human genetic diseases (reviewed in Tronchere H. et al., 2003).

In conclusion, we used the TAP technique to identify interacting partners of the MMR proteins hMLH1 and hPMS2. This technique proved to be a valuable tool that allowed us to validate known interactions and to discover new potential binding partners. The biological significance of the identified interactions will be evaluated for a selection of potentially interesting molecules that will hopefully help us to better understand the MMR mechanism and/or to discover novel functions of hMutLα in the cell.

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FIGURE LEGENDS

Fig.1 Characterization of mammalian cell lines stably expressing TAP-tagged hMLH1 and hPMS2

A: Whole cell extracts of TAP-hMLH1/293T (left panel) and TAP-hPMS2/HeLa12 cells (right panel) were analyzed by Western blot (50 μg of extract/lane) for the expression of hMLH1 and hPMS2. In both cell lines, the expression of the TAP-tagged proteins and their respective major interacting partners is comparable to the amounts of these proteins present in 50 μg of whole cell extract from the MMR-proficient cell line HeLa (first lane). Note that TAP-hMLH1 and TAP-hPMS2 migrate slower due to the presence of the TAP-tag.

B: *In vitro* MMR assays. The repair efficiency of the extracts of TAP-hMLH1/293T (left panel) and TAP-hPMS2/HeLa12 (right panel) cells was compared with the repair efficiency of the extracts from corresponding parental MMR-deficient cell lines 293T or HeLa12. The repair efficiencies were determined on hetroduplex substrates containing a G/T mismatch (see *Methods* for details). Extracts from the MMR-proficient HeLa cells were used as a positive control.

C: FACS profiles of the MMR-proficient or -deficient HeLa or HeLa12 cells, (left panel), and 293 and 293T (right panel) were compared with the profile of TAP-hMLH1/293T (left panel) and TAP-hPMS2/HeLa12 (right panel) cells either untreated or treated with 0.2 μM of MNNG for 24 hours.

Fig.2 Tandem Affinity Purification strategy

A: Schematic representation of the hMLH1- (upper panel) and hPMS2-TAP (lower panel) constructs. The TAP-tag was inserted at the N-terminus of hMLH1 and at the C-terminus of hPMS2. Prot A, Protein A (IgG binding) domain; TEV, TEV protease cleavage site; CBD, Calmodulin Binding Domain.

B: Overview of the purification procedure. Black circles represent factors that specifically interact with the bait protein and white circles represent non-interactors (see text for details).

Fig.3 Analysis of hMLH1- and hPMS2-interacting partners by TAP

A: Analysis of the TAP-hMLH1 (right lane, left panel) and TAP-hPMS2 (right lane, right panel) interactors. TAP with extracts from corresponding parental untransfected cells (middle lane) was used as a negative control. One third of the final eluate from 60 mg whole cell extract (see *Methods* for details) was resolved on a SDS-polyacrylamide gel and visualized by silver staining. The bands corresponding to the tagged protein and its major *in vivo* interactor are indicated. M; Molecular size marker, 20 ng/band. CBD; Calmodulin Binding Domain.

B: Western blot analysis. 50 μg of whole cell extract (WCE) or 33 μl of TAP eluate were loaded on a SDS-polyacrylamide gel and analyzed by Western blotting using specific antibodies against human hMLH1 and hPMS2. Extracts and eluates from parental untransfected cell lines 293T (left panel) and HeLa12 (right panel) were compared with samples from the stable cell lines TAP-hMLH1/293T (left) and TAP-hPMS2/HeLa12 (right). Note that the TAP-tagged hMLH1 and hPMS2 migrate according to the presence of the TAP-tag or of the Calmodulin Binding Domain (CBD).

Fig.4 Relative abundance of hPMS1 and hPMS2 in TAP eluates

A: Silver stained SDS-PAGE gel of hMLH1-TAP eluate. The band corresponding to hPMS1 has comparable intensity to the band corresponding to hPMS2. M: Molecular size marker, 20 ng/band. CBD: Calmodulin Binding Domain.

B: Western blot analysis. 50 μg of whole cell extract (WCE) from 293T and TAP-hMLH1/293T cells or 33 μl of the final eluate from the TAP were loaded on SDS-

polyacrylamide gel and analyzed by Western blotting using specific antibodies against human hPMS1 and hPMS2.

Fig.5: Co-immunoprecipitation of hPMS1-interacting partners from HeLa cell extracts

A: Example of large-scale co-immunoprecipitation analysis of hPMS1. Immunoprecipitation was performed with 5 mg of whole cell extract and 1 μg of affinity-purified anti-hPMS1 rabbit polyclonal antibody or purified pre-immune serum. Immunoprecipitates were analyzed by SDS-PAGE and visualized by Coomassie staining. M: Molecular size marker, 2 μg/band.

B: Western blot analysis of hPMS1 immunoprecipitates. Only the purified anti-hPMS1 antibody and not the pre-immune serum efficiently immunoprecipitates hPMS1 and hMLH1.

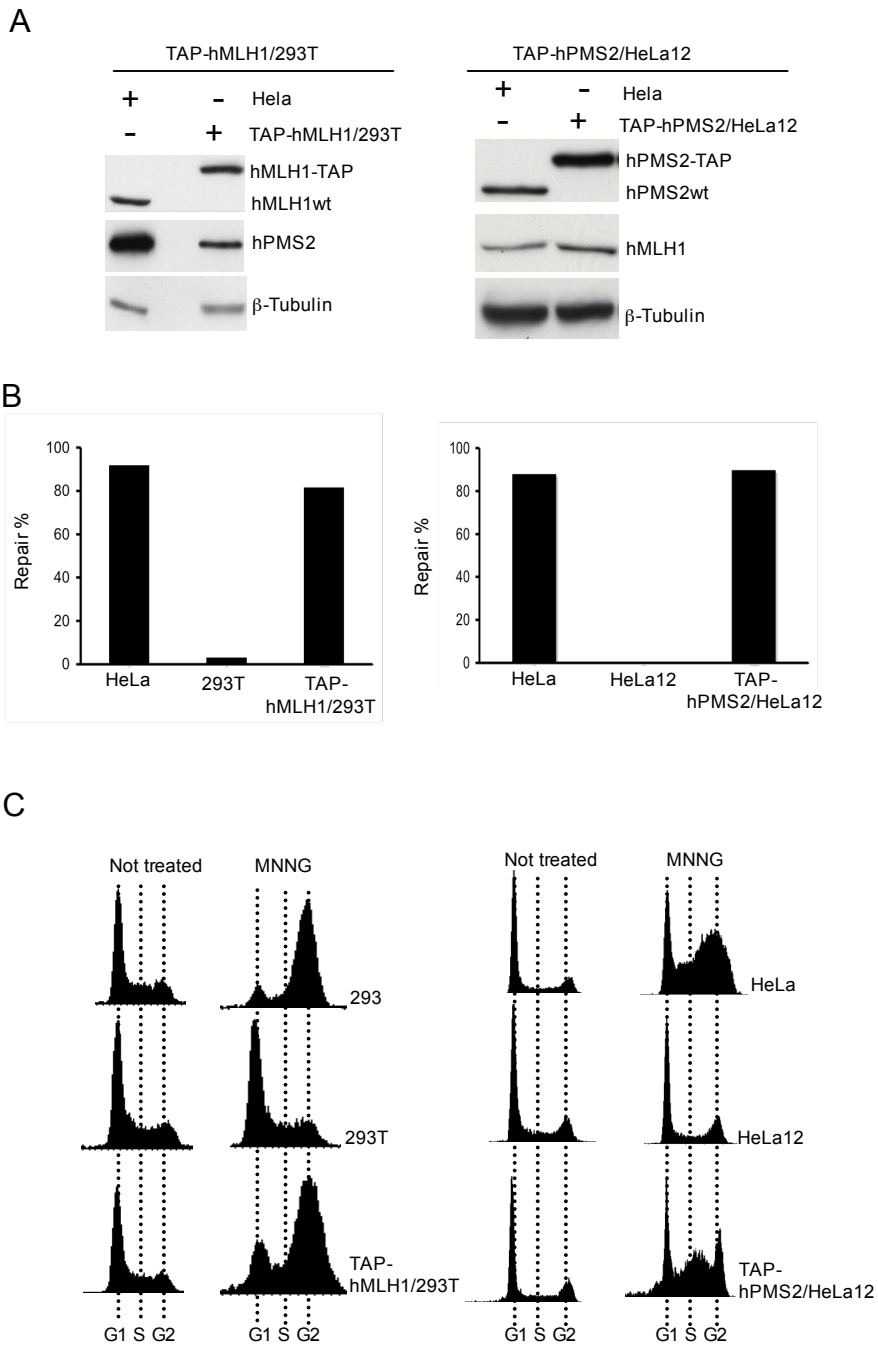
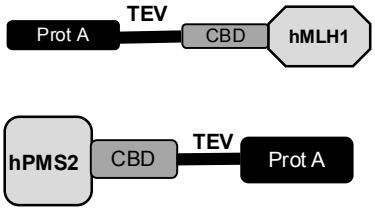

A
TAP-hMLH1/293T
TAP-hPMS2/HeLa12
+ - Hela
- + TAP-hMLH1/293T
hMLH1-TAP
hMLH1wt
hPMS2
β-Tubulin
+ - Hela
- + TAP-hPMS2/HeLa12
hPMS2-TAP
hPMS2wt
hMLH1
β-Tubulin
B
Repair %
100
80
60
40
20
0
HeLa
293T
TAP-hMLH1/293T
Repair %
HeLa
HeLa12
TAP-hPMS2/HeLa12
C
Not treated
MNNG
293
293T
TAP-hMLH1/293T
G1 S G2
Not treated
MNNG
HeLa
HeLa12
TAP-hPMS2/HeLa12
G1 S G2

Fig.1

A


Prot A
TEV
CBD
hMLH1
hPMS2
CBD
TEV
Prot A

B

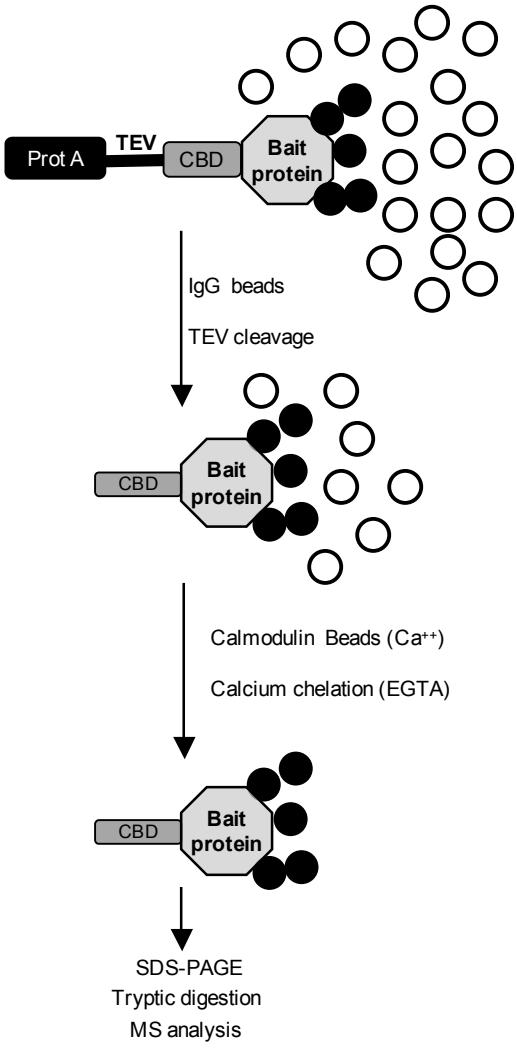

Prot A
TEV
CBD
Bait protein
IgG beads
TEV cleavage
CBD
Bait protein
Calmodulin Beads (Ca++)
Calcium chelation (EGTA)
CBD
Bait protein
SDS-PAGE
Tryptic digestion
MS analysis

Fig.2

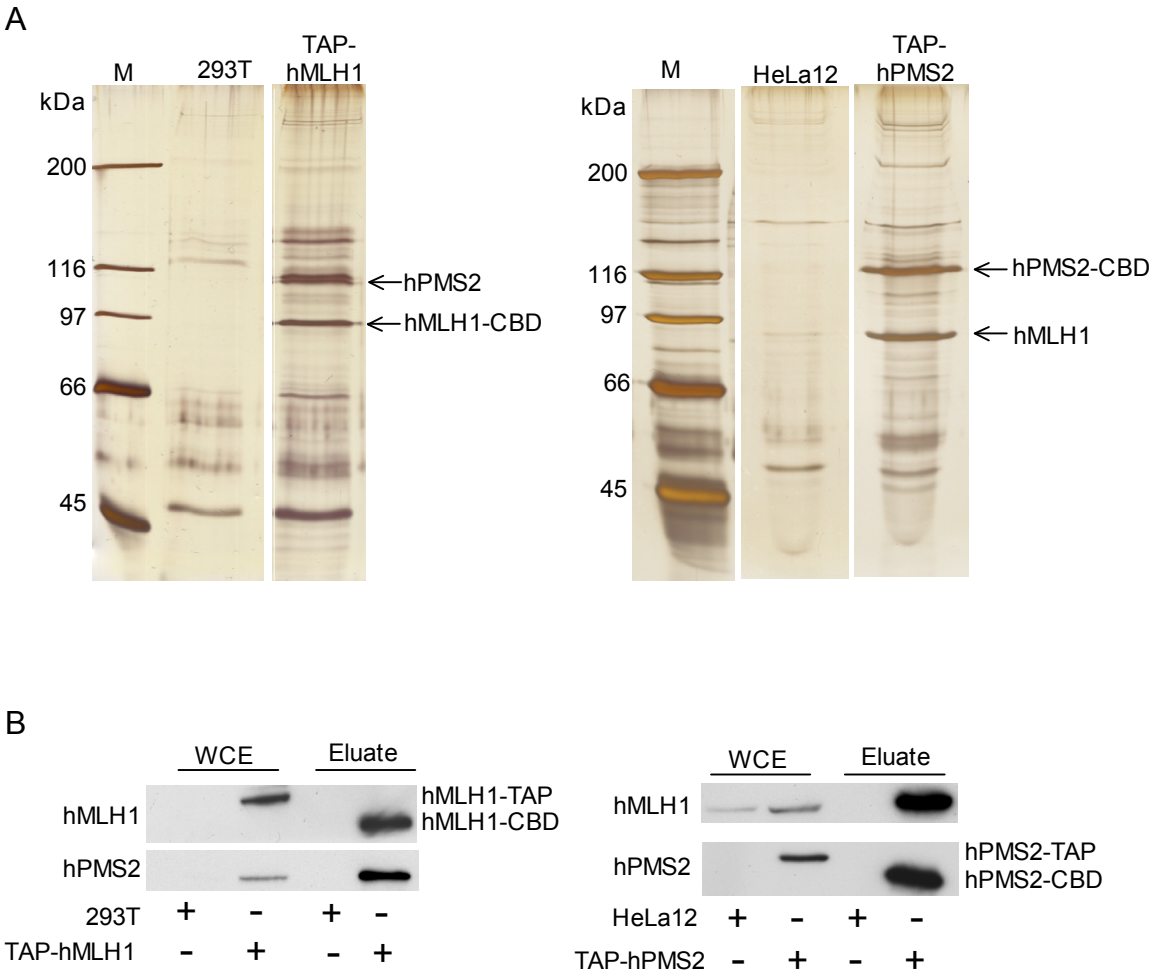

A
M
293T
TAP-
hMLH1
kDa
200
116
97
66
45
←hPMS2
←hMLH1-CBD
M
HeLa12
TAP-
hPMS2
kDa
200
116
97
66
45
←hPMS2-CBD
←hMLH1
B
WCE
Eluate
hMLH1
hPMS2
hMLH1-TAP
hMLH1-CBD
293T + - + -
TAP-hMLH1 - + - +
WCE
Eluate
hMLH1
hPMS2
hPMS2-TAP
hPMS2-CBD
HeLa12 + - + -
TAP-hPMS2 - + - +

Fig.3

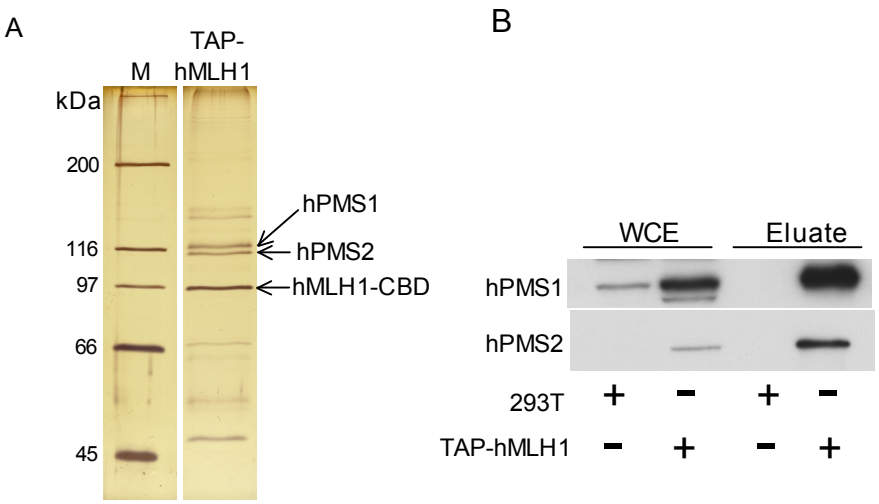

A
M
TAP-hMLH1
kDa
200
116
97
66
45
hPMS1
hPMS2
hMLH1-CBD
B
WCE
Eluate
hPMS1
hPMS2
293T
TAP-hMLH1

Fig.4

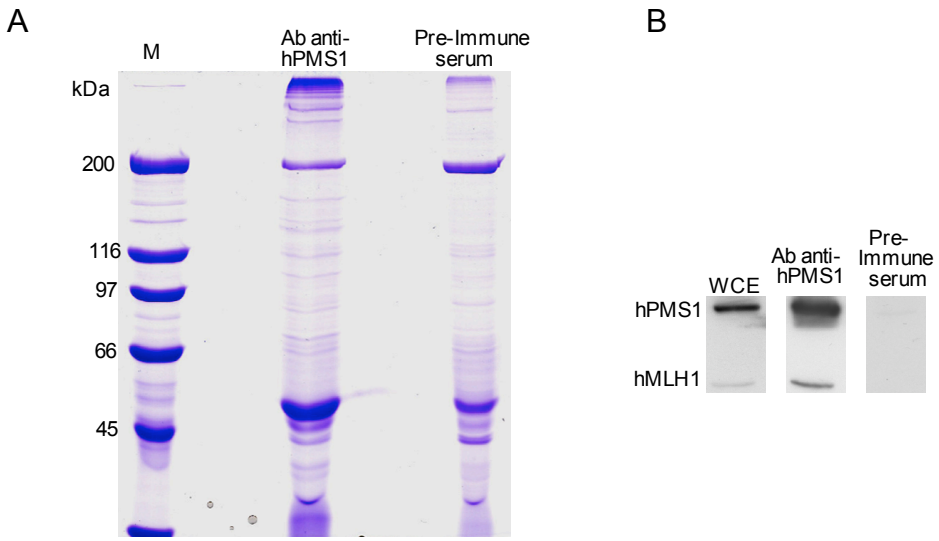

A
M
Ab anti-
hPMS1
Pre-Immune
serum
kDa
200
116
97
66
45
B
WCE
Ab anti-
hPMS1
Pre-
Immune
serum
hPMS1
hMLH1

Fig.5

Table1: Proteins associated with hMLH1. Shown is a selection of proteins present specifically in hMHL1-TAP eluate.

Function^	Protein	Protein score*	Sequence coverage (%)	Swiss-Prot acc.no.
Mismatch repair				
	hMLH1	7390	70	P40692
	hPMS1	7143	70	P54277
	hPMS2	4117	59	P54278
	hMSH3	628	22	P20585
	Exonuclease 1	72	6	Q5T396
Other repair Pathways				
	DNA-PKcs	483	9	P78527
	BRCA1	202	11	Q5YLB2
	SMC1A	68	10	Q14683
Proteins Import/Export				
	Importin alpha2	336	18	P52292
	Importin beta 1	118	3	Q14974
	Importin alpha 4	108	5	O00629
	Importin alpha 6	70	8	O15131
Ubiquitin pathway/proteasome				
	PSD3	243	18	O43242
	UBP2L	183	12	Q14157
	Ubiquitin	172	45	P62988
	IFP38	66	3	Q9BX72
DNA Helicases				
	BRIP1(BACH1)	3905	53	Q9BX63
	SEP1(XRN1)	330	9	Q8IZH2
	RuvB like1	309	22	Q9Y265
	RuvB like2	76	8	Q9Y230
Heat Shock Proteins/Chaperones				
	Hsp60	1169	48	P10809
	TCPA (TCP-1 sub.alpha)	456	23	P17987
	TCPD (TCP-1 sub.delta)	192	10	P50991
	Hsp90alpha(Hsp86)	94	11	P07900
Unknown Function/Hypotetical proteins				
	KIAA1018(fragment)	898	27	Q9Y2M0
	YLPM1 (ZAP3)	184	3	P49750
	AN11H (WDR68)	95	5	P61962
	FLJ10178	89	9	Q9NWA6
	KIAA0553	73	5	Q9UKJ3

Table1: continued

Function^	Protein	Protein score*	Sequence coverage (%)	Swiss-Prot acc.no.
Metabolism of RNA/Transcription/Translation/Ribosome				
	EF1-alpha2	456	25	Q05639
	hnRNP A1	260	15	P09651
	WUGSC:H_RG054D04.1	201	14	O95036
	EF-Tu	137	12	P49411
	RLA0	173	16	P05388
	RS3A	166	18	P61247
	eIF3-p47	163	8	O00303
	DDX3Y	151	10	O15523
	DDX17	145	8	Q92841
	RL6	134	15	Q02878
	RL4	132	16	P36578
	eIF3-p167	120	7	Q14152
	RNMT	109	10	O94996
	PheRS (SYFA)	97	9	Q9Y285
	CPSF6	91	5	Q9BSJ7
	hnRNP K	86	11	P61978
	PRP4	85	6	O43172
	SF3B-1 (SAP155)	85	5	O75533
	SFRS3(SRP20)	79	18	P78406
	RAE1L	85	6	P84103
	ILF2(NF45)	84	11	Q7L7R3
	NPM1(Numatrin)	74	12	P06748
	eIF3-p66	72	6	O15371
	PABPL1	66	10	Q9NTZ0
Cell cycle/Signaling/Kinases/Phospatases/Apoptosis				
	PP2A reg. sub A- alpha	278	19	P30153
	PP2A reg. sub B-alpha	152	10	P63151
	PP2A reg. sub B-beta	99	6	Q00005
	P2BB catalytic sub-beta	184	19	P16298
	P2BC catalytic sub-gamma	122	10	P48454
	PP2A reg. sub. B-delta	99	8	Q6IN90
	PDCD8	169	15	O95831
	PI3K-C2alpha	160	6	O00443
Others				
	Angiomotin	4013	57	Q4VCS5
	ATAD3A	470	25	Q9NVI7
	DOCK7	460	9	Q5T1C0
	PYGB	269	10	P11216
	ATPalpha	953	39	P25705
	ATAD3B	244	19	Q5T9A4
	REC14(WDR61)	155	7	Q6IA22
	HDAC2	88	12	Q92769
	YTHDF3	82	14	Q7Z739
	ARMCX3(ALEX3)	72	13	Q9UH62
	NCOA3(TRAM1)	70	5	Q9Y6Q9

^ Derived from Swiss-Prot. database or published data.
* Mascot protein score where a value >65 was considered significant ($P<0.05$).

Table2: Proteins associated with hPMS2. Shown is a selection of proteins present specifically in hPMS2-TAP eluate.

Function^	Protein	Protein score*	Sequence coverage (%)	Swiss-Prot acc.no.
Mismatch repair				
	hMLH1	4158	85	P40692
	hPMS2	3601	71	P54278
	hMSH2	1529	31	P43246
	hMSH3	993	24	P20585
	hMSH6	108	4	P52701
	Exonuclease 1	164	12	Q5T396
	PCNA	184	15	P12004
	RFC 40kDa	82	10	P35250
Other repair Pathways				
	DNA-PKcs	1938	13	P78527
	BRCA2	114	8	P51587
	DDB1	114	8	Q16531
	MMS19-like	97	97	Q5T455
Proteins Import/Export				
	Importin alpha2	535	22	P52292
	Importin beta 1	425	12	Q14974
	Importin beta3	87	10	O00410
	CRM1 (XPO1)	797	17	O14980
	COPB	741	24	P53618
	COPG	170	4	Q9Y678
	COPG2	112	7	Q9UBF2
Ubiquitin pathway/proteasome				
	PSD2	818	24	Q13200
	PRS4	378	29	P62191
	PRS10	253	14	P62333
	PSD5	245	16	Q16401
	PSD3	234	14	O43242
	PRS6A	132	12	P17980
	PRS7	108	7	P35998
	Ubiquitin	127	45	P62988
	CYLD	241	10	Q9NQC7
	EDD	206	8	O95071
	HERC2	99	3	O95714
	PRS8	73	8	P62195
DNA Helicases				
	BRIP1(BACH1)	720	14	Q9BX63
	RuvB like1	710	33	Q9Y265
	RuvB like2	570	24	Q9Y230
Heat Shock Proteins/Chaperones				
	Hsp90alpha(HSP86)	496	16	P07900
	Hsp70/7	193	18	P48741
	Hsp60s2	173	22	Q96RI3
	Hsp60s1	127	20	Q96RI4
	Hsp75	91	2	Q12931
	HSP60	954	30	P10809
	Hsp70/2	745	21	P54652
	TCPA (TCP-1 sub.alpha)	359	15	P17987
	SSRA	164	12	P43307
	TCPG (TCP-1 sub.gamma)	105	4	P49368
	STIP1(HOP)	75	14	P31948
Replication/DNA metabolism				
	CAD(PYR1)	1718	19	P27708
	MCM3	130	7	P25205

Table2: continued

Function^	Protein	Protein score*	Sequence coverage (%)	Swiss-Prot acc.no.
Unknown Function/Hypotetical proteins				
	KIAA1018(fragment)	454	14	Q9Y2M0
	DKFZp686L22104	103	21	Q68E03
	FLJ16757	91	8	Q6ZMQ9
	KIAA1541	88	10	Q9P1Y7
	FLJ26613	87	5	Q6ZP32
	DKFZp667O1117	85	5	Q5JPJ1
	DKFZp586H1322	81	7	Q9H065
	KIAA1729	73	11	Q9C0D4
	DKFZp451I106	70	12	Q86T41
	DKFZp434P055	70	13	Q8NCX0
Metabolism of RNA/Transcription/Translation/Ribosome				
	EF-Tu	413	24	P49411
	hnRNP M	360	15	P52272
	GCN1L	348	9	Q92616
	RLA0	329	21	P05388
	SMCA4	280	5	P51532
	EF2	272	10	P13639
	EEF1A	209	14	Q14222
	PABP1	189	15	P11940
	RL18	183	21	Q07020
	RS3a	185	26	P61247
	PABP3	152	11	Q9H361
	RL7	137	14	P18124
	WUGSC:H_RG054D04.1	129	15	O95036
	RL4	101	14	P36578
	Exportin T	385	11	O43592
	DSRAD	93	6	P55265
	U5S1	93	6	Q15029
	DDX39	92	8	O00148
	PRP4	83	11	O43172
	hnRNP E2	80	10	Q15366
	GRIPE	72	3	Q6GYQ0
	eIF2-gamma	66	6	P41091
	RRP44	146	8	Q5W0P7
	ABCE1	134	13	P61221
	DHX15	175	7	O43143
	DDX46	72	11	Q7L014
Cell cycle/Signaling/Kinases/Phospatases/Apoptosis				
	RIF-1 isoform 4	97	3	Q5UIP0
	PDCD8	90	6	O95831
	PP2A reg.sub B-beta	105	6	Q00005
	PP2A catalitic sub-alpha	131	12	P67775
	PI3K-C2alpha	67	9	O00443
Others				
	ATAD3A	571	30	Q9NVI7
	NSUN2	145	10	Q9BVN4
	SMRA3(HIP116)	74	9	Q14527

^ Derived from Swiss-Prot. database or published data.
* Mascot protein score where a value >65 was considered significant ($P<0.05$).

Table3: Proteins associated with hPMS1. Shown is a selection of proteins that specifically immunoprecipitate with the anti-hPMS1 antibody.

Function^	Protein	Protein score*	Sequence coverage (%)	Swiss-Prot acc.no.
Mismatch repair				
	hMLH1	3692	74	P40692
	hPMS1	3118	70	P54277
	hMLH3	94	8	Q9UHC1
	RFC 40kDa	120	18	P35250
	RFC 37kDa	111	11	P35249
	RFC 140kDa	98	6	P35251
	Exonuclease 1	65	10	Q5T396
	RPA 40kDa	83	12	O15160
Other repair Pathways				
	BRCA2	224	10	P51587
	BRCA1	78	6	P38398
	MMS19-like	103	14	Q5T455
	XPF	83	14	Q92889
	ATR	157	9	Q13535
	NONO	283	28	Q9BQC5
	ATM	99	7	Q13315
	RAD18	86	23	Q9NS91
	RAD54B	76	13	Q9Y620
Proteins Import/Export				
	Importin beta3	851	24	O00410
	Importin alpha 2	312	23	P52292
	Importin 9	296	11	Q96P70
	RANBP9	338	20	Q96S59
	RANGAP1	243	29	Q96JJ2
	Importin 3	184	12	O14787
Ubiquitin pathway/proteasome				
	EDD	2558	34	O95071
	UBP5	963	35	P45974
	CYLD	183	7	Q9NQC7
	UBP13	141	15	Q92995
	Ubiquitin	111	58	P62988
	RNF123	103	7	Q5XPI4
	UBAP2L	101	7	Q9BTU3
	PSD2	506	23	Q13200
	PRS4	198	16	P62191
	Herc2	213	8	O95714
	Cullin 3	190	15	Q13618
	Cullin 1	134	11	Q13616
	USP9Y	102	6	O00507
	RNF20	188	14	Q5VTR2
	UBP26	83	15	Q9BXU7

Table3: continued

Function^	Protein	Protein score*	Sequence coverage (%)	Swiss-Prot acc.no.
DNA Helicases				
	Dna helicase B	706	23	Q8NG08
	MOV10	330	14	Q9HCE1
	BRIP1	159	15	Q9BX63
	RECQ4	95	8	O94761
Heat Shock Proteins/Chaperones				
	HSP90beta	2299	63	P08238
	TCPA (TCP-1sub.alpha)	543	42	P17987
	Midasin	345	5	Q9NU22
	HSP75	192	16	Q12931
	Sacsin	182	9	Q5T9J7
Replication/DNA metabolism				
	PGK1	487	30	P00558
	TOP1	403	18	P11387
	DPOD1	169	11	P28340
	MCM6	278	12	Q14566
	DPOZ	229	8	O60673
	POLQ	139	9	Q6VMB5
	Dna Pol. Gamma sub. 1	77	4	P54098
	SMC3	170	17	Q9UQE7
Unknown Function/Hypotetical proteins				
	WDHD1(AND-1)	537	21	O75717
	RO52 (TRIM21)	513	33	P19474
	MARE2	482	55	Q15555
	DKFZp686E0722	377	13	Q68DX7
	KIAA1018 (fragment)	349	17	Q9Y2M0
	KIAA0889	295	10	Q6ZTG8
	KIAA1401	285	15	Q9P2E6
Metabolism of RNA/Transcription/Translation/Ribosome				
	EF1-delta	517	49	P29692
	RLA0	512	50	P05388
	SK2L2	505	18	P42285
	hNRP U	1052	40	Q9BQ09
	U520	504	16	O75643
	SR140	401	15	O15042
	ABCF1	398	31	Q8NE71
	RS8	369	50	P62241
	U2AF2	362	30	P26368
	eIF-5B	284	17	O60841
	hnRNP M	218	18	P52272
	eIF-2B	140	8	Q13144
	eIF-4B	254	12	P23588
	UBF-1	280	20	P17480
	DHX36	261	12	Q8IYE5
	PABPC4	261	16	Q5SPS6
	TRRAP	224	14	Q9Y4A5
	ASH1	217	10	Q9NR48
	EXOSC10	213	17	Q01780
	RRBP1	183	16	Q9P2E9
	POLR1A	157	12	O95602

Table3: continued

Function^	Protein	Protein score*	Sequence coverage (%)	Swiss-Prot acc.no.
Cell cycle/Signaling/Kinases/Phospatases/Apoptosis				
	SET binding factor 2	759	17	Q86WG5
	SET binding factor 1	194	8	O95248
	Cyclin T1	483	30	O60563
	CDK9	444	38	P50750
	PI3K-C2 alpha	443	15	O00443
	CENP E	384	19	Q02224
	CENP F	263	12	P49454
	CENP J	212	15	Q5T6R5
	CDC5-like	338	22	Q76N46
	AKAP9	323	15	Q99996
	PP2A reg. sub. A-alpha	263	22	P30153
	PP2A reg. sub. A-beta	113	18	P30154
	CDC42BPA	127	7	Q5VT25
	CK5P2	189	12	Q96SN8
	NEK1	150	15	Q96PY6
	PKCB	125	18	Q9ULU4
Others				
	ACC-alpha	459	16	Q7Z561
	ATAD 34	362	25	Q5SV23
	DPH5	370	36	Q9H2P9
	Dnmt1	120	9	P26358
	APC	198	11	P25054
	HRMT1L2	175	19	Q99873
	SUP6H	175	9	Q7KZ85
	Angiomotin	82	12	Q4VCS5

^ Derived from Swiss-Prot. database or published data.
* Mascot protein score where a value >65 is considered significant ($P<0.05$).

4.3.
Methylation-induced G(2)/M arrest requires a full complement of the mismatch repair protein hMLH1.

Cejka P, Stojic L, Mojas N, Russell AM, Heinimann K, Cannavo E, di Pietro M, Marra G, Jiricny J.

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Methylation-induced G_2/M arrest requires a full complement of the mismatch repair protein hMLH1

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The mismatch repair (MMR) gene *hMLH1* is mutated in ~50% of hereditary non-polyposis colon cancers and transcriptionally silenced in ~25% of sporadic tumours of the right colon. Cells lacking hMLH1 display microsatellite instability and resistance to killing by methylating agents. In an attempt to study the phenotypic effects of hMLH1 downregulation in greater detail, we designed an isogenic system, in which hMLH1 expression is regulated by doxycycline. We now report that human embryonic kidney 293T cells expressing high amounts of hMLH1 were MMR-proficient and arrested at the G_2/M cell cycle checkpoint following treatment with the DNA methylating agent *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG), while cells not expressing hMLH1 displayed a MMR defect and failed to arrest upon MNNG treatment. Interestingly, MMR proficiency was restored even at low hMLH1 concentrations, while checkpoint activation required a full complement of hMLH1. In the MMR-proficient cells, activation of the MNNG-induced G_2/M checkpoint was accompanied by phosphorylation of p53, but the cell death pathway was p53 independent, as the latter polypeptide is functionally inactivated in these cells by SV40 large T antigen.

Keywords: cell cycle checkpoint/hMLH1/methylating agent/mismatch repair/TetOff

Introduction

Mutations in mismatch repair (*MMR*) genes, predominantly *hMSH2* and *hMLH1*, segregate with hereditary non-polyposis colon cancer (HNPCC). Inheritance of a single mutated allele of a *MMR* gene predisposes to precocious cancers of the colon, endometrium and ovary. Analysis of HNPCC tumour cells showed that repeated sequence elements (microsatellites) in their genomic DNA are frequently mutated (for a review see Peltomaki, 2001). As microsatellite instability (MSI) is a hallmark of defective MMR in all organisms tested to date, and has been shown to be present in all tumour cell lines that have lost both alleles of *hMSH2* or *hMLH1* (Boyer *et al*., 1995), it is assumed that the wild type alleles of the respective *MMR* genes in cells of HNPCC tumours have been lost or inactivated by mutation. But mutations in *MMR* genes are not an absolute prerequisite for MSI. In recent years, a number of sporadic colon tumours and tumour cell lines displaying MSI have been described that are MMR-deficient due to silencing of the *hMLH1* promoter by hypermethylation (reviewed in Esteller, 2002).

Once both *MMR* gene alleles have been inactivated, the cell's propensity towards acquiring mutations increases, especially in genes carrying microsatellite repeats. Should the mutated genes be involved in the control of cell proliferation, the mutator cell in, for example, the colonic epithelium would be able to divide in an uncontrolled manner and thus give rise to an adenomatous polyp. As the cells in this benign growth acquire further mutations with subsequent cell divisions, the adenoma would rapidly become transformed into a carcinoma. That such a path to transformation can be followed *in vivo* was demonstrated when numerous HNPCC colon cancers were shown to carry frameshift mutations in a run of 10 adenines within the coding sequence of the transforming growth factor β receptor type II (*TGFβRII*) gene, as well as in other genes involved in growth control or apoptosis (reviewed in Markowitz *et al*., 2002). Further support for this hypothesis comes from the finding that adenomas of HNPCC kindred transform to carcinomas with a much higher frequency than those associated with sporadic disease (Kinzler and Vogelstein, 1998), presumably due to a more rapid acquisition of transforming mutations.

The above findings help explain how the loss of MMR might accelerate cellular transformation and tumour progression. What is unclear to date, however, is whether the transformation process begins only following the inactivation of both *MMR* gene alleles, or whether it commences already at the stage when only one allele is affected or when the expression of the given *MMR* gene is only attenuated, rather than shut off, such as might be the case in cells where the *hMLH1* promoter is only partially methylated. The notion that a reduction in MMR protein levels might promote tumorigenesis originates in studies with *Msh2*$^{+/-}$ mice. Although the *Msh2*$^{+/-}$ embryonic stem cells were apparently normal in terms of their MMR capacity as measured by MSI (de Wind *et al*., 1995), the heterozygous animals were cancer prone, and presented with tumours that often still contained the wild-type *Msh2* allele (de Wind *et al*., 1998). The propensity of the *MMR* heterozygous cells to transformation would thus appear to be linked to a process distinct from the correction of replication errors. What might the nature of these processes be?

In recent years, MMR defects have been linked to several other phenomena, such as transcription-coupled repair and recombination—both mitotic and meiotic (reviewed in Harfe and Jinks-Robertson, 2000). In

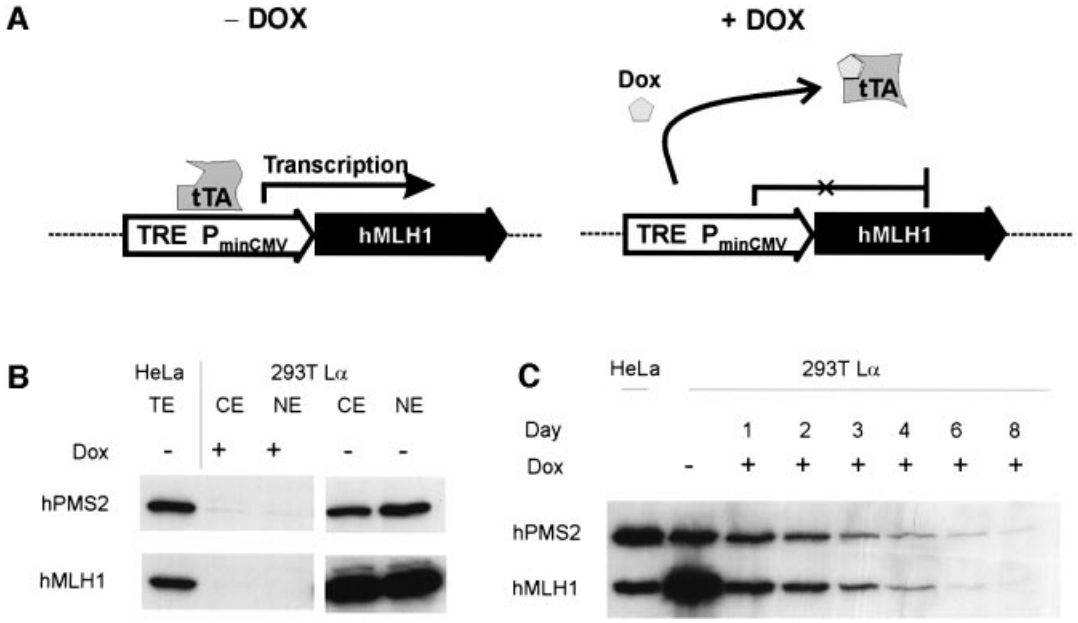


Fig. 1. Inducible *hMLH1* expression in 293T Lα cells. (**A**) In the Tet-Off system, hMLH1 is expressed in the absence of Dox, because the tTA factor binds to the promoter of the expression vector and thus activates transcription. Addition of Dox to the culture medium causes a conformational change in tTA, which leads to its dissociation from the promoter and thus to an inactivation of *hMLH1* transcription. (**B**) Western blot analysis of cytoplasmic (CE) and nuclear (NE) extracts of cells cultured in the absence (–) or presence (+) of 50 ng/ml Dox. hMLH1 and hPMS2 were visualized using anti-hMLH1 or anti-hPMS2 antibodies as described in Materials and methods. Total extract (TE) of MMR proficient HeLa cells was used as a positive control. (**C**) Stability of hMutLα. The cells were cultured without Dox (–) to induce maximal hMLH1 expression. Following the addition of 50 ng/ml Dox (+), total cell extracts were isolated after 1, 2, 3, 4, 6 and 8 days. Western blot analysis was performed using anti-hMLH1 and anti-hPMS2 antibodies as in (B).

addition, the MMR system was implicated in activation of cell cycle checkpoints and apoptosis, as witnessed by the increased resistance of MMR-deficient cells to the methylating agent *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) or cisplatin (reviewed in Bellacosa, 2001). Thus, while $MMR^{+/-}$ cells, or cells expressing low amounts of MMR proteins, may not display a mutator phenotype, they might have at least a partial defect in one of the above processes, specifically in the DNA damage signalling pathway, which we judged to be of the greatest relevance to cancer. We wanted to study these processes in detail, but we lacked isogenic cells expressing varying amounts of MMR proteins. Cells in which the MMR defect was corrected either by transfer of a chromosome carrying a single wild-type copy of the mutated *MMR* gene (Koi *et al*., 1994) or its cDNA (Risinger *et al*., 1998; Buermeyer *et al*., 1999; Lettieri *et al*., 1999; Claij and Te Riele, 2002) were unsuitable for our studies, because they express similar or even higher amounts of the complementing MMR proteins than MMR-proficient controls. Thus, in order to be able to study the phenotypic consequences associated with reduced levels of MMR proteins, we had to generate a new line, preferably of epithelial origin, in which the expression of a selected *MMR* gene could be regulated. We now describe the construction and characterization of a line in which the expression of *hMLH1* can be tightly regulated by doxycycline with the help of the TetOff system.

Results

Construction of cells with inducible hMLH1 expression

The human embryonic kidney cell line 293T is MMR deficient, because the *hMLH1* gene in these cells is epigenetically silenced by promoter hypermethylation (Trojan *et al*., 2002). We set out to correct its MMR defect through the expression of exogenous hMLH1 using the TetOff expression system, which can be tightly regulated. We first generated the 293T-TetOff cell line by stable transfection of the 293T cells with a DNA vector encoding the tetracycline-controlled transactivator (tTA). In the second step, we stably transfected the 293T-TetOff cells with a vector carrying the *hMLH1* cDNA under the control of the tetracycline response element (TRE), thus creating 293T Lα cells. In the absence of tetracycline, or its more stable analogue doxycycline (Dox), the tTA protein binds to the TRE and activates transcription of *hMLH1*; conversely, addition of the drug induces a conformational change in tTA, which loses its ability to bind DNA and the transcription of *hMLH1* is thus turned off (Figure 1A). During the initial screening, we used Dox at a concentration of 2 μg/ml, as recommended by the manufacturer, but later we found that a concentration of 50 ng/ml was sufficient to turn off the expression of hMLH1 below the limit of detection by western blotting (see below).

In vivo, hMLH1 interacts with hPMS2 to form the heterodimer hMutLα, which is essential for MMR. Our previous studies have shown that hPMS2 is unstable in the absence of its cognate partner (Räschle *et al*., 1999). Indeed, no hMLH1 could be detected in the extracts of 293T cells, and hPMS2 was hardly detectable (Trojan *et al*., 2002). A similar situation also existed in our 293T Lα clone grown in the presence of Dox, i.e. under conditions where the *hMLH1* promoter is shut off (Figure 1B). However, expression of hMLH1 brought about hPMS2 stabilization through the formation of hMutLα, such that the levels of the latter protein were

comparable to those seen in extracts of MMR-proficient cell lines (Figure 1B).

The expression of hMLH1 in the 293T Lα cells grown in the absence of Dox was substantially higher than in any MMR-proficient cell line tested by us to date (Figure 1B; data not shown). Interestingly, this overexpression did not appear to be toxic to the cells: we detected no increase in the rates of apoptosis, as described for cells microinjected with expression vectors encoding hMSH2 and hMLH1 (Zhang *et al*., 1999). Moreover, cells grown in the absence or presence of Dox divided roughly once every 24 h (data not shown), unlike HCT116 and SNU-1 cells, in which the stable expression of hMLH1 was reported to result in substantially slower growth rates (Shin *et al*., 1998). When the expression of the transgene was turned off by the addition of Dox, the hMLH1 and hPMS2 proteins were present in the cell extracts in a 1:1 ratio only 24 h later (Figure 1C) and decayed with similar kinetics. This experiment showed that hMutLα is extremely stable, as it was detectable in the extracts of 293T Lα cells even 6 days after the expression of hMLH1 was shut off.

In the following text, cells grown in the presence of 50 ng/ml Dox that do not express hMLH1 and thus lack hMutLα will be referred to as 293T Lα$^-$ cells. Those grown in the absence of Dox, which express hMLH1 and thus contain functional hMutLα, will be referred to as 293T Lα$^+$ cells.

hMLH1 expression in 293T Lα cells restores MMR in vitro

Extracts of the 293T Lα cells were tested for MMR activity *in vitro* using two different MMR assays (see Materials and methods). No MMR activity was detected in extracts of 293T Lα$^-$ cells, but as the defect could be complemented by the addition of the recombinant wild-type hMutLα, we concluded that this heterodimer was the only factor missing in these extracts (Figure 2). In contrast, extracts from 293T Lα$^+$ cells were MMR proficient in both assays (Figure 2). Importantly, these results showed that the excess partnerless hMLH1 in the 293T Lα line does not inhibit MMR, at least not in our *in vitro* system. This differs from the situation in *Saccharomyces cerevisiae*, where overexpression of MLH1 gave rise to a mutator phenotype associated most likely with the inhibition of MMR through the homodimerization of this polypeptide (Shcherbakova and Kunkel, 1999; Shcherbakova *et al*., 2001). The MMR proficiency of the 293T Lα$^+$ cells in our *in vitro* assay was similar irrespective of whether the extracts were prepared from cells grown in the absence of Dox, or 24 h after the addition of the drug (data not shown), at which time point the ratio of hMLH1 to hPMS2 was 1:1 (Figure 1C).

Inducible hMLH1 expression restores sensitivity to alkylating agents

In order to determine the effect of hMLH1 expression on the sensitivity of 293T Lα cells to MNNG, we used clonogenic assays to quantify the surviving fraction of 293T Lα$^-$ and 293T Lα$^+$ cells following treatment with 5 μM MNNG. [Note that 293T Lα cells do not express MGMT, the enzyme responsible for the detoxification of methylation damage (G.Marra, unpublished data). For this reason, the experiments described below were carried out

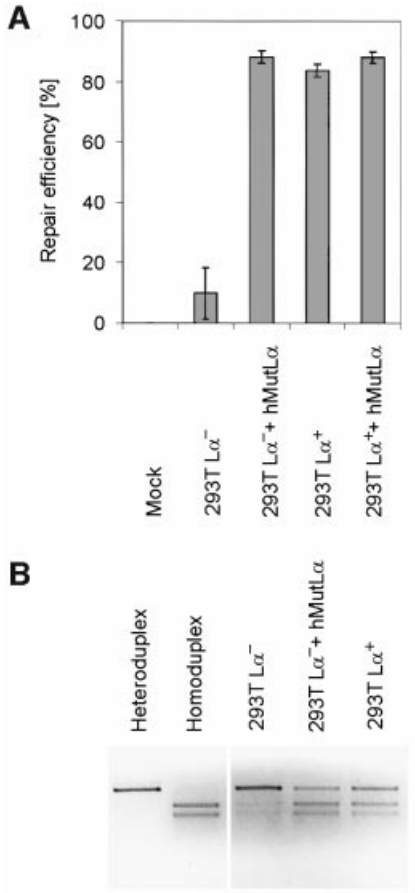
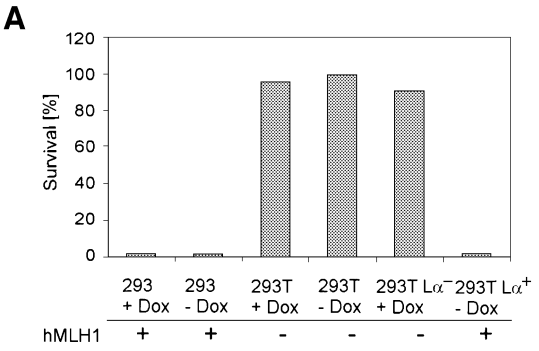


Fig. 2. MMR proficiency of 293T Lα cell extracts. (**A**) Repair efficiency of a G/T mismatch in the M13mp2 vector carrying a strand discrimination signal 3′ from the mispair, using cytoplasmic extracts of the 293T Lα$^+$ and 293T Lα$^-$ cells, supplemented or not with recombinant hMutLα (see text for details). Error bars show standard errors. (**B**) Correction of a G/T mismatch within a *Bgl*II restriction site of a pGEM vector, following incubation with nuclear extracts of 293T Lα$^+$ or 293T Lα$^-$ cells, supplemented or not with recombinant hMutLα. The strand discrimination signal in this heteroduplex substrate was 5′ from the mispair. Efficient repair resulted in the restoration of a *Bgl*II site and in the generation of two DNA fragments that co-migrate with those observed in the reference digest of the homoduplex molecule carrying a bona fide *Bgl*II site.

in the absence of the MGMT inhibitor O^6-benzylguanine.] As shown in Figure 3A, 293T Lα$^+$ cells were very sensitive to killing by MNNG, and the surviving fraction was indistinguishable from that obtained after MNNG treatment of the related MMR-proficient 293 cell line. In contrast, 293T Lα$^-$ cells were resistant to killing by MNNG, just like the parental, MMR-deficient 293T cells. The presence of Dox in the culture medium had no effect on the survival of any of the control cell lines used in this study (Figure 3A).

The sensitivity of 293T Lα cells to MNNG was further examined using the MTT assay, which is based on the cleavage of the yellow tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] by the action of mitochondrial dehydrogenases to form a violet formazan dye. As this reaction takes place only in living cells, these can be distinguished from non-viable cells in a simple colorimetric assay. As shown in Figure 3B, 293T Lα$^-$ cells were 125-fold more resistant



B

	IC_{50} [μM MNNG]
293T Lα$^+$	0.05 ± 0.003
293T Lα$^-$	6.26 ± 0.500

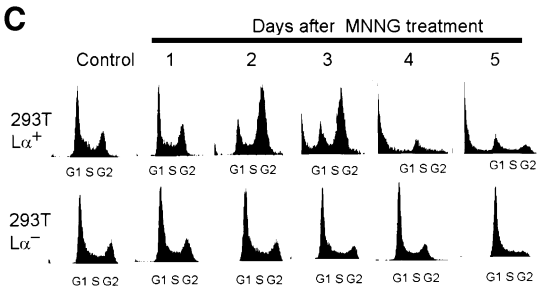


Fig. 3. Sensitivity of 293T Lα cells to MNNG. (**A**) Survival of 293T Lα$^+$ and 293T Lα$^-$ cells following treatment with 5 μM MNNG. 293 and 293T cells were used as MMR-proficient and -deficient controls, respectively. The presence of Dox (+Dox) in the culture medium did not affect the control cells, but had a dramatic effect on the survival of the 293T Lα cell populations. (**B**) IC_{50} values of 293T Lα$^+$ and 293T Lα$^-$ cells. Each value represents the mean ± SE. (**C**) Cell cycle profiles of 293T Lα$^+$ and 293T Lα$^-$ cells treated with 0.2 μM MNNG. Shown are representative cytometrograms of cells expressing (293T Lα$^+$) and not expressing (293T Lα$^-$) hMLH1. G_1, cell population in the G_1 phase of the cell cycle with a 2n DNA content; G_2, cells in the G_2 and M stages of the cell cycle with a 4n DNA content; S, cells in various stages of DNA synthesis with a DNA content between 2n and 4n.

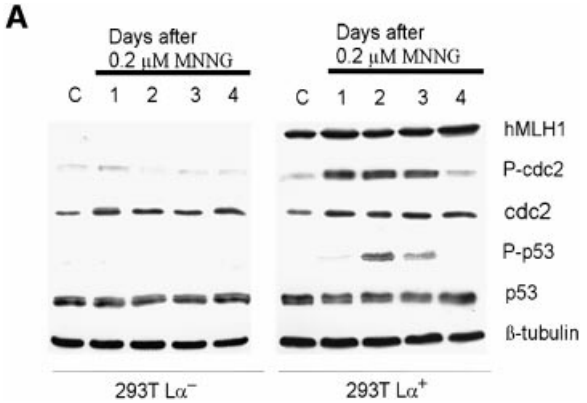
to killing by MNNG than the same cells in a MMR-proficient mode (i.e. 293T Lα$^+$ cells).

Expression of hMLH1 in 293T Lα cells leads to activation of a methylation damage induced cell cycle arrest

To determine whether the increased sensitivity of 293T Lα$^+$ cells to MNNG resulted from induction of cell cycle arrest and cell death, the treated 293T Lα$^+$ and 293T Lα$^-$ cell populations were analysed by flow cytometry. As shown in Figure 3C, 2 days after treatment with 0.2 μM MNNG, the 293T Lα$^+$ cells were mostly arrested in the G_2/M phase of the cell cycle. One day later, cells containing sub-G_1 amounts of DNA became detectable, and this population increased with time. In contrast, no increase in the population of cells either arrested in G_2/M or with a lower than 2n DNA content was detected in cultures of treated 293T Lα$^-$ cells.

In order to further characterize the response of cells to MNNG, we analysed the phosphorylation status of Cdc2. As shown in Figure 4A, Cdc2 phosphorylated on Tyr15 accumulated exclusively in 293T Lα$^+$ cells treated with 0.2 μM MNNG. This provides molecular evidence for a G_2/M arrest, because so long as this kinase remains phosphorylated, entry into mitosis should be blocked. No difference in Cdc2 phosphorylation was observed in the extracts of MNNG-treated 293T Lα$^-$ cells (Figure 4A).

The above results thus show that induction of hMLH1 expression in the 293T Lα cells was necessary and sufficient to endow them with a MMR-proficient status, which also enabled them to respond to DNA damage induced by MNNG. What is presently unclear is the role of the MMR system in this checkpoint activation. DNA damage signalling is known to be mediated via several protein phosphorylation cascades, which involve primarily the DNA-dependent protein kinase (DNA-PK), or the ataxia telangiectasia-mutated (ATM) and ATM and Rad3-related (ATR) kinases. The downstream target of the latter enzymes is the p53 tumour suppressor protein, the phosphorylation of which on Ser15 is known to lead to its stabilization and subsequent activation as a transcription factor (Tibbetts *et al*., 1999). Phosphorylation of p53 has indeed been shown to take place upon MNNG treatment, and was shown to be dependent on functional hMutSα and hMutLα (Duckett *et al*., 1999; Hickman and Samson, 1999; Adamson *et al*., 2002). However, as the latter experiments were carried out with drug concentrations 25- to 125-fold higher than those used in our study, we wanted to test whether Ser15 phosphorylation also took place in the 293T Lα cells treated with 0.2 μM MNNG. These cells overexpress the SV40 large T antigen and thus contain large amounts of stabilized p53 polypeptide. This system is ideally suited for the study of post-translational modification of p53, as the steady-state levels of the latter protein remain unaltered during the experiment (Tibbetts *et al*., 1999). As anticipated, the p53 steady-state levels in the 293T Lα cell extracts were high, irrespective of whether hMLH1 was expressed or not, or whether extracts of treated or untreated cells were examined (Figure 4A). However, following MNNG treatment, phosphorylation of p53 with a Ser15-specific antibody could be detected exclusively in the MMR-proficient 293T Lα$^+$ cells. Notably, and in contrast to the study by Adamson *et al*. (2002), where the phosphorylation of p53 became detectable already just minutes after MNNG treatment, the MMR-dependent post-translational modification of p53 observed in our cells peaked at 48 h, i.e. at a time point where most cells were arrested at G_2/M (Figure 3C). This difference is probably linked with the high concentration of MNNG (25 μM) used in the latter study, which would be expected to introduce numerous single- and double-strand breaks into DNA that arise through the spontaneous loss of methylated purines and the subsequent breakage of the sugar-phosphate DNA backbone by β-elimination at the resulting abasic sites (Loeb, 1985). DNA strand breaks rapidly activate the ATM/ATR kinases that subsequently phosphorylate a number of downstream targets, one of which is histone H2AX (Redon *et al*., 2002). This histone modification is thought to aid the recruitment of DNA repair factors to the sites of damage (Paull *et al*., 2000). H2AX is phosphorylated in the 293T Lα cells upon



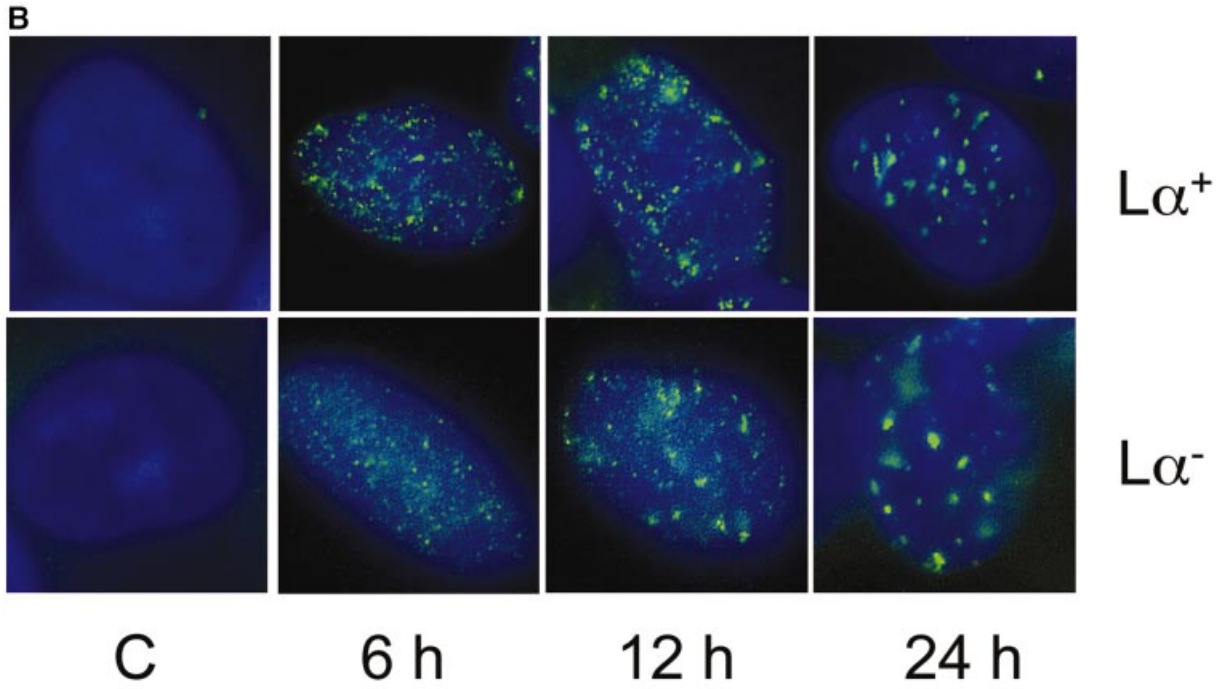


Fig. 4. Post-translational protein modification and strand break processing in MNNG-treated 293T Lα cells. (**A**) Phosphorylation status of p53 and cdc2 in 293T Lα$^+$ and 293T Lα$^-$ cells 1–4 days after treatment with 0.2 μM MNNG. P-p53, P-cdc2, phosphorylated p53 and cdc2 proteins, respectively; C, untreated control cells; β-tubulin, internal standard used to ascertain equal gel loading. (**B**) γ-H2AX foci formation in MNNG-treated 293T Lα cells. In the control cell population, <10% of cells displayed H2AX foci. Following MNNG treatment, all cells contained foci until 24 h post-treatment. See text for details and Materials and methods for experimental procedures.

treatment with 0.2 μM MNNG, as witnessed by the formation of phospho-H2AX foci (Figure 4B). However, these foci arise in both 293T Lα$^+$ and 293T Lα$^-$ cells soon after treatment. Thus, damage caused by direct modifications of DNA at low concentrations of MNNG does not trigger the G_2/M checkpoint. The activation of the checkpoint machinery must take place after H2AX phosphorylation, in the second cell cycle post-treatment (Kaina *et al*., 1997), and must involve the MMR system, perhaps in conjunction with another pathway of DNA metabolism that remains to be identified. Thus, the lesions that trigger the checkpoint machinery are distinct from those that bring about phosphorylation of H2AX.

MMR proficiency and response to MNNG treatment require different levels of hMLH1 expression

The principal goal of this study was to investigate the phenotypic effects of reduced expression of MMR proteins, such as might be encountered when expression of the gene is attenuated by cytosine methylation. In order to achieve this goal, we attempted to modulate hMLH1 expression in the 293T Lα cells. This could be achieved by varying the Dox concentration in the culture media. Thus, cells grown in the presence of 0.1, 0.2, 0.4, 0.8 and 1.5 ng/ml Dox contained steadily decreasing amounts of hMLH1 and hPMS2, as compared with cells grown in the absence of the drug (Figure 5A).

When we tested how this variation in the amount of hMutLα affected MMR efficiency, we found that extracts of cells expressing as little as 10% of the amounts found in cells grown in the absence of Dox were still proficient in the *in vitro* MMR assays. Cells cultivated with 0.1 and 0.2 ng/ml Dox showed MMR activities comparable to those of the MMR-positive 293T Lα$^+$ cells grown in the absence of Dox, and even extracts of cells cultivated with 0.4 ng/ml Dox were still able to repair mismatches *in vitro*, albeit with lower efficiency (Figure 5B). MMR proficiency was lost only in cell extracts in which the hMLH1 and hPMS2 proteins became difficult to detect by western blotting (Figure 5A). To test whether the results of the *in vitro* MMR assays were reflected also in the MSI

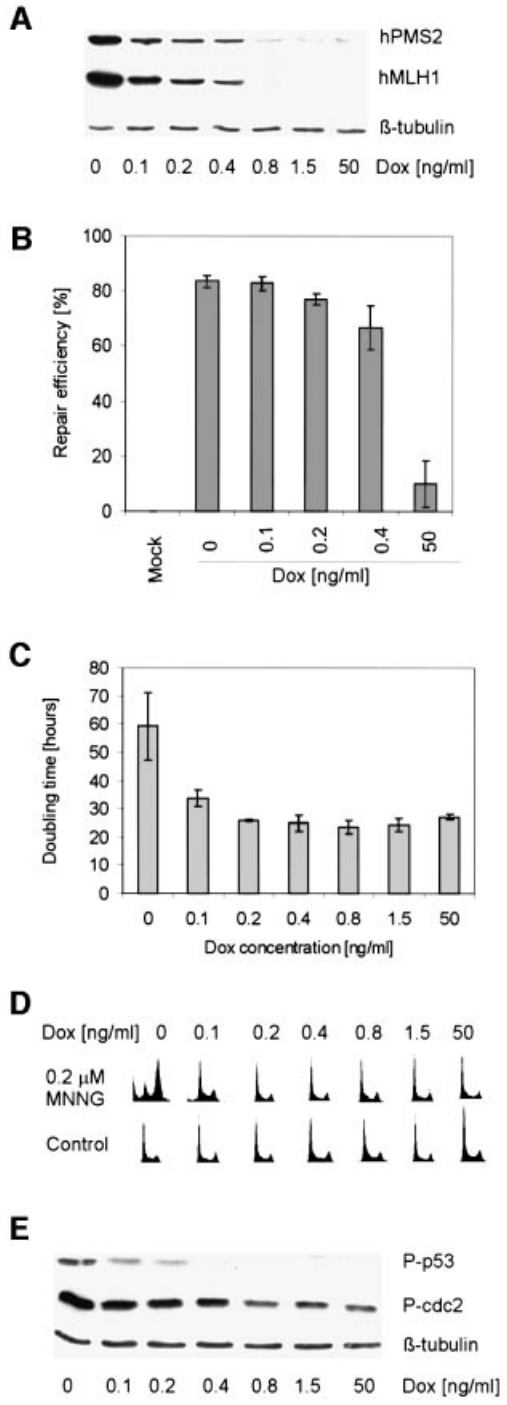


Fig. 5. Mismatch correction efficiency and MNNG-induced G_2/M arrest in cells expressing different amounts of hMLH1. (**A**) Dependence of hMLH1 expression on Dox concentration. hMLH1 and hPMS2 were visualized as described in Materials and methods. β-tubulin, internal standard used to ascertain equal loading. (**B**) MMR efficiency of a G/T mispair in an M13mp2 substrate carrying a strand-discrimination signal 3′ from the mispair. Error bars show standard errors. (**C**) Variation in doubling times of 293T Lα cells grown in the indicated Dox concentrations following treatment with 5 µM MNNG. (**D**) FACS analysis of 293T Lα cell populations grown in the indicated Dox concentrations, either untreated (Control), or 72 h after treatment with 0.2 µM MNNG (see also Figure 3C). (**E**) Phosphorylation of p53 and cdc2 48 h after treatment of cells (grown in the indicated Dox concentrations) with 0.2 µM MNNG. β-tubulin, internal standard used to ascertain equal loading.

Table I. Instability of the BAT26 chromosomal locus in 293T Lα cells expressing varying amounts of hMLH1

Dox (ng/ml)	MSI+/total	% MSI
0	2 (0)/131	1.5
0.2	1 (0)/80	1.3
50	4 (2)/73	5.5 (2.7)

MSI+ clones were defined as those displaying more than three extra peaks in the sequence of the PCR product. Numbers in parentheses refer to clones with more than four extra peaks.

phenotype of the cells, we analysed the BAT26 microsatellite marker, which contains a repeat of 26 deoxyadenosines, and which is considered to be a reliable indicator of MSI. Because the 293T Lα cells are hypotriploid, and because this cell line was MMR deficient for many generations prior to our intervention, the BAT26 locus was found to be highly heterogeneous. The product of PCR amplification had on average eight peaks, and we therefore applied the HNPCC criteria of MSI (Loukola *et al.*, 2001), whereby only PCR products that differed by three or more peaks at this locus were considered to be a sign of MSI. By these criteria, the BAT26 instability in the cells propagated for 35 generations in 0 or 0.2 ng/ml Dox was ~1%, whereas cells grown with 50 ng/ml Dox displayed MSI that was ~5-fold higher (Table I). However, closer inspection of the data revealed that cells propagated in 0 or 0.2 ng/ml Dox displayed no alleles (0/211) that differed by more than 4 bp from the median. In contrast, two such alleles (two out of 73; 2.7%) were found in the cells grown with 50 ng/ml Dox (Table I, numbers in parentheses). This suggests that MSI at the BAT26 locus in the 293T Lα⁻ cells is substantially greater than in cells expressing hMLH1, and thus that expression of even low amounts of hMutLα are sufficient to correct the MMR defect in these cells, both *in vitro* (Figure 2) and *in vivo* (Table I).

We were interested to determine whether the low amounts of the hMLH1/hPMS2 heterodimer that were shown to restore MMR proficiency were also able to activate the DNA damage-induced cell cycle arrest in 293T Lα cells. To this end, we treated the cells with 5 µM MNNG and calculated the average doubling time over a period of 5 days. In accordance with our previous experiments, only cells expressing the highest amounts of hMLH1 (i.e. 293T Lα+ cells grown without Dox) ceased to grow, as suggested by their increased doubling time. Cells grown in 0.1 ng/ml Dox were only partially affected, and cells cultivated with 0.2 ng/ml Dox or more grew similarly to 293T Lα⁻ cells (Figure 5C). To test whether this growth retardation was due to checkpoint activation, we analysed the DNA content of the cells 3 days after treatment with 0.2 µM MNNG. As shown in Figure 5D, FACS analysis showed that only cells expressing the highest amounts of hMLH1 (i.e. cells cultured without Dox) displayed a strong G_2/M arrest (an average of 63% of the cells were in G_2/M). The response of cells cultivated with 0.1 ng/ml Dox was substantially weaker (~27% cells in G_2/M), and the cell cycle profiles of cells grown with 0.2 ng/ml Dox or more were indistinguishable from those of the untreated controls (~22% cells in G_2/M). Notably, whereas cells grown in the absence of Dox activated the MNNG-induced G_2/M checkpoint, while those grown in

0.2 ng/ml Dox failed to do so, phosphorylated forms of p53 and cdc2 could be detected in both cell populations (Figure 5E). The extent of cdc2 phosphorylation in particular would predict that a detectable proportion of the treated cells should be at the G_2/M boundary. This was clearly not the case, as judged by FACS analysis (Figure 5D; also see Figure 3C).

Taken together, these experiments show that although only low amounts of hMutLα are required for MMR proficiency, DNA damage-induced G_2/M arrest and cell death in response to MNNG treatment require a full complement of this heterodimer. The fact that the 293T Lα$^+$ cells arrest and die with kinetics and efficiency similar to other MMR-proficient cells confirms that p53, which is inactive in these cells, is not required for either of these processes (Hickman and Samson, 1999). Thus, the molecular pathways controlling the MNNG-induced G_2/M checkpoint in these cells require further study.

Discussion

We show that expression of hMLH1 in 293T Lα cells corrected their MMR defect *in vitro* and *in vivo*. The 293T Lα$^+$ cells were also found to be >100-fold more sensitive to killing by MNNG than the isogenic cells lacking hMLH1. MNNG treatment arrested the MMR-proficient cells in the G_2/M phase of the cell cycle, and this arrest was entirely and solely dependent on the function of hMLH1. This latter statement is supported by the finding that expression of hMLH1 in 293T Lα$^+$ cells did not affect the transcriptional activity of other genes, as demonstrated by Affymetrix GeneChip™ analysis (data not shown).

This study also showed that the steady-state levels of the hMLH1/hPMS2 heterodimer required for MMR proficiency and DNA damage response were significantly different. In earlier experiments (Lettieri *et al*., 1999) we generated a cell line derived from hMSH6-deficient HCT15 cells, which expressed low levels (~20%) of wild-type hMSH6. This line was MMR proficient, but remained as resistant to killing by methylating agents as the parental cell line. Similarly, a recent study described a *Msh2*$^{-/-}$ mouse embryonic stem cell line in which the MMR defect was largely corrected by the expression of low levels (10% of control) of exogenous Msh2, but the response of these cells to methylating agents was comparable to that observed with the parental *Msh2*$^{-/-}$ cells (Claij and Te Riele, 2002). This damage signalling defect was suggested by the authors to be linked to poor recognition of ^{Me}G/T mispairs, which arise through the mispairing of O^6-methylguanine (^{Me}G) with thymine during DNA replication (Karran and Bignami, 1996), and which are bound less efficiently than bona fide mispairs by the hMSH2/hMSH6 (hMutSα) heterodimer (Duckett *et al*., 1996). Constant loading of hMutSα sliding clamps at ^{Me}G/T mispairs was proposed be responsible for transmission of the DNA damage signal to the checkpoint machinery *in vivo* (Fishel, 1999), and it might be expected that this process is substantially less efficient in cells expressing only low amounts of the mismatch binding factor hMutSα. However, the amounts of hMutSα in 293T Lα$^+$ and 293T Lα$^-$ cells are equal, and similar to those found in other MMR-proficient cells. Our results thus extend the above hypothesis by showing that the signal transduction process also requires the hMLH1/hPMS2 heterodimer, thought to act downstream of damage recognition. Moreover, our result show that the recognition of ^{Me}G/T mispairs *per se* is insufficient to activate the checkpoint machinery. The G_2/M checkpoint is thought to be controlled by the phosphoinositide-3 (PI3) kinases ATM/ATR, which are principally responsible for the phosphorylation of p53 on Ser15 (Osborn *et al*., 2002). The ^{Me}G/T mispairs arise already during the first round of replication, yet no p53 phosphorylation is detectable until 24 h after treatment, at which point the cells are beginning to enter the second S phase (Figure 3C; data not shown). Notably, the peak of signalling activity coincides with that of chromosomal rearrangements (sister chromatid exchanges and recombinations) induced by MNNG (Kaina *et al*., 1997). Thus, MMR-dependent processing of the ^{Me}G/T mispairs that arise during the first S phase apparently does not activate the checkpoint machinery, but leads instead to the generation of intermediates that result in aberrant recombination events during the subsequent round of DNA replication, which then signal. What the exact nature of these intermediates may be is currently the subject of intensive studies.

The evidence presented here shows that cells with lower than wild-type levels of MMR proteins are not phenotypically normal, despite being MMR proficient. The observed defect in DNA damage signalling may be relevant to cellular transformation and cancer, particularly in epithelial cells that are rapidly proliferating and that may be exposed to stress or carcinogens. In the colon, the epithelial stem cells that are near the bottom of the crypts give rise to daughter cells that begin to differentiate during their migration towards the surface of the colon. Upon reaching the apex of the crypt, these cells undergo apoptosis and are shed. When the colonic epithelial cells become damaged, they should undergo apoptosis and thus give rise to no mutant progeny. In contrast, cells with a defect in DNA damage signalling, such as those expressing suboptimal amounts of MMR proteins, would not activate cell cycle checkpoints and apoptosis in response to DNA damage. Instead, they might acquire mutations that allow them to continue to proliferate and give rise to an adenoma.

The relevance of this hypothesis to the situation *in vivo* hinges on two points. First, there are currently no experimental data documenting instances where colonocytes or other epithelial cells that are prone to transformation express low MMR protein levels. We obtained some evidence of lower than normal steady-state levels of hMSH2 and increased resistance to methylating agents in the immortalized lymphoblasts of HNPCC patients, which are heterozygous in the *hMSH2* locus, but the *hMLH1*$^{-/-}$ cells were normal in all assays (Marra *et al*., 2001). It is not known whether hMSH2 and hMLH1 levels in heterozygous colonocytes of HNPCC kindred are lower than in similar cells of normal individuals, even though some fluctuations might be expected. However, the recent characterization of the *hMLH1* promoter as a frequent target of DNA hypermethylation (Esteller, 2002) implies that there must be cells with only partially methylated promoters, because *de novo* methylation of CpG islands is a gradual process. These cells, such as the 293T Lα cells grown in low concentrations of Dox (Figure 5A), would

contain decreased levels of hMutLα and would therefore be likely to also have a defective response to DNA damage.

The second point concerns the nature of the endogenous DNA damage that might trigger the transformation process. It is conceivable that normal colonocytes which become damaged by endogenous or exogenous DNA modifying agents would arrest and, in cases where the extent of the damage is beyond repair, activate cell death processes, while those expressing reduced levels of hMutLα would continue to proliferate and thus acquire mutations. Although human DNA is aberrantly modified by *S*-adenosyl methionine and other methyl group donors, the extent of such modifications might be too low to trigger cell death. However, the deleterious effects of the checkpoint defect could become evident also in response to other types of DNA damage; experimental evidence implicates the MMR system in the processing of DNA modifications ranging from oxidative damage to bulky moieties such as cisplatin and AAF (reviewed in Bellacosa, 2001).

We have described a cell line in which the MMR status can be controlled by the concentration of doxycycline in the culture medium. Our current results show that the activation of transcription of exogenous *hMLH1* complements not only the MMR defect of the 293T cells, but also reactivates their responsiveness to treatment with methylating agents, providing that the levels of the MMR proteins are sufficiently high to activate the DNA damage-induced checkpoint. This fully isogenic system is clearly open to further exploitation, and should allow us to study the involvement of the MMR system in other pathways of DNA metabolism, such as response to other types of DNA damaging agents ranging from ionizing radiation to crosslinking chemotherapeutics, where the involvement of MMR was found to be only marginal and where it could not be ruled out that the observed effects (or lack thereof) were linked to a selection of an atypical clone from the stably transfected population. The 293T Lα line could also be used in the screening for substances that preferentially kill MMR-deficient cells. This should prove invaluable in the treatment of tumours, both hereditary and sporadic, with defective MMR.

Materials and methods

Cell lines

The 293T cells (a kind gift of K.Ballmer) were grown in Dulbecco's modified Eagle's medium with Eagle salts (Gibco-BRL, Gaithersburg, MD), supplemented with 10% Tet System Approved Fetal Bovine Serum (Clontech, Palo Alto, CA), 2 mM L-glutamine (Gibco-BRL), 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco-BRL). For 293T-TetOff or 293T Lα cells, 100 μg/ml Zeocin (Invitrogen, San Diego, CA) or 100 μg/ml Zeocin and 300 μg/ml Hygromycin B (Roche Molecular Biochemicals, Mannheim, Germany) were added, respectively.

Plasmid construction

The pTetOff-Zeo plasmid was constructed by ligation of the following DNA molecules: the first, coding for tTA, was obtained by digestion of pTetOff (Clontech) with *Xho*I (New England Biolabs, Beverly, MA) followed by filling-in with dCTP and dTTP using the Klenow fragment of DNA polymerase I (New England Biolabs). The second, coding for Zeocin resistance protein, was obtained by digestion of pVgRXR (Invitrogen) with *Bam*HI (New England Biolabs) followed by filling-in with dGTP and dATP. The pTRE2-hMLH1 plasmid was generated by subcloning hMLH1 cDNA (a kind gift of R.Michael Liskay) into the *Bam*HI and *Not*I sites of pTRE2 (Clontech).

Calcium phosphate transfections

One day before transfection, 250 000 cells were plated in 6-well plates in 3 ml of cell culture medium. The cells reached ~50% confluency on the day of transfection. Three hundred microlitres of solution A (250 mM $CaCl_2$) was carefully mixed with 15 μg DNA and 300 μl of solution B (140 mM NaCl, 50 mM HEPES, 1.4 mM Na_2HPO_4 pH 7) in an Eppendorf tube. Exactly 1 min after mixing, 300 μl of the precipitation cocktail was added to the medium. The plates were incubated for 4 h at 37°C. The medium was then removed, the cells were washed with phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4{\cdot}7H_2O$, 1.4 mM KH_2PO_4) and fresh cell culture medium was added.

Generation of the 293T Lα cell line

293T cells were transfected with pTetOff-Zeo using the calcium phosphate method (see above). The selection of stable cell lines was initiated 2 days later using 400 μg/ml Zeocin. After 3 weeks, ~50 colonies were isolated and screened by transient transfection with pTRE2-Luc (Clontech) for the expression of luciferase in induced and noninduced cells (with or without 2 μg/ml Dox; Clontech). The clone with the lowest background and high induction of luciferase (293T-TetOff) was then transfected with pTRE2-hMLH1 and pTK-Hyg (ratio 15:1). Selection of stable cell lines was initiated 2 days post-transfection using 400 μg/ml hygromycin-B. After 3 weeks, ~160 colonies were isolated and their extracts were screened by western blotting using antibodies against hMLH1, hPMS2 and β-tubulin. The clone 293T Lα was selected for further study, as it displayed the highest induction of hMLH1 in the absence of Dox, and no background expression with 2 μg/ml Dox.

Regulation of hMLH1 expression

293T Lα cells were grown in the presence of 50 ng/ml Dox to keep hMLH1 expression turned off; fresh Dox was added every second day. To induce hMLH1 expression, the cells were transferred to a Dox-free medium, and the cells were cultivated for at least 6 more days. To obtain cells completely free of hMLH1, cells grown in the absence of Dox were kept for a least 7 days in a medium containing 50 ng/ml Dox. To obtain intermediate levels of hMLH1, the cells were cultivated with 1.5, 0.8, 0.4, 0.2 or 0.1 ng/ml Dox for at least 7 days.

Preparation of total protein extracts for western blots

Cells were harvested, transferred to a 1.5 ml Eppendorf tube and washed twice with PBS. Cell lysis was performed on ice in 50 mM Tris–HCl pH 8, 125 mM NaCl, 1% NP-40, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1× complete protease inhibitory cocktail (Roche Molecular Biochemicals) for 25 min. Insoluble material was pelleted by centrifugation at 18 000 *g* for 3 min at 2°C. Protein concentration was determined using the Bradford assay (Bio-Rad, Munich, Germany).

Western blot analyses

The primary antibodies used in this study were: anti-hMLH1 [PharMingen, San Diego, CA), 1:2000 in TBST (20 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.1% Tween-20 with 2.5% non-fat dry milk], hPMS2 (Calbiochem; 1:500), β-tubulin, p53 (Santa Cruz Biotechnology; 1:1500 and 1:2000, respectively), cdc2 (Upstate Biotechnology; 1:1000) and phospho-p53-Ser15, phospho-cdc2-Tyr15 (Cell Signalling Technology; 1:1000 and 1:5000, respectively). The proteins (20–50 μg) were denatured, reduced, separated by SDS–PAGE (7.5–12.5%) and transferred to Hybond-P PVDF membrane (Amersham Pharmacia Biotech) according to standard protocols (Sambrook *et al*., 1989). The membranes were blocked with 5% non-fat dry milk in TBST for 60 min, incubated with primary antibodies for 60 min, washed three times with TBST for 10 min, incubated with the peroxidase-conjugated secondary antibody (anti-mouse IgG, 1:5000 in TBST with 2.5% non-fat dry milk) for 60 min and washed three times with TBST for 10 min. Immunoreactive proteins were detected using enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech).

Indirect immunofluorescence experiments

Cells grown on coverslips were treated or mock-treated with MNNG (0.2 μM end concentration) and incubated for 6, 12 and 24 h (Figure 4B). Foci of phosphorylated histone H2AX were visualized using an anti-phospho-H2AX rabbit polyclonal antibody (Upstate Biotechnology) at +4°C, over night, at a dilution of 1:100. The procedure was as described previously (Kleczkowska *et al*., 2001). To allow direct comparisons, all

the cells were treated and processed simultaneously, and all the images were obtained using the same magnification, brightness and contrast settings.

MMR assays

The cell extracts were prepared as described previously (Marra *et al*., 2001; Nystrom-Lahti *et al*., 2002). Two different *in vitro* assays were used. The first, adapted from Holmes *et al*. (1990), is based on a circular 3′ 193 bp DNA molecule containing a G/T mismatch within a unique *Bgl*II recognition site, a single-strand nick 369 nucleotide residues 5′ from the mismatch in the G-containing strand, and a unique *Bsa*I site. This molecule is refractory to cleavage with *Bgl*II, unless the mispair is corrected to an A/T. Thus, the unrepaired heteroduplex digested with both endonucleases gives rise to only a single fragment of 3′ 193 bp, whereas the repaired homoduplex is cleaved into two fragments of 1′ 833 and 1′ 360 bp (Nystrom-Lahti *et al*., 2002).

The second method, originally described by Thomas *et al*. (1991), makes use of an M13mp2 DNA heteroduplex containing G/T mismatch within lacZα complementation gene, obtained by hybridizing single-stranded viral (+) DNA with the replicative form I (–) strand. The repair is directed to the (–) strand by the presence of a nick. The method was described in detail elsewhere (Marra *et al*., 2001). In the complementation studies, extracts were supplemented with purified recombinant hMutLα (0.1 μg).

MTT assays

Two thousand cells/well were plated in 96-well plates, treated the next day with various concentrations of MNNG (Sigma; diluted in dimethyl sulfoxide and stored at –20°C in the dark) and incubated for 5 days. Then, 20 μl of MTT solution (5 mg/ml MTT; Sigma; in PBS, sterile filtered) was added, and the plates were incubated for 4–5 h at 37°C. One volume of lysis solution was then added (20% SDS, 50% dimethylformamide pH <4.7), and the plates were incubated overnight at 37°C. The solubilized formazan was quantified at 570 nm, using the Versamax microplate reader (Molecular Devices, Sunnyvale, CA). The optical density values were plotted against logarithm of MNNG concentrations and IC_{50} values were calculated from the regression curve.

Colony-forming assays

Cells in log phase (50–80% confluent) were treated with 5 μM MNNG, harvested after 2 h, and 200 or 2000 cells per duplicate were plated in 10 cm plates. Colonies were counted after 15–20 days of incubation. Survival was calculated as the ratio of the number of colonies from treated versus untreated samples.

Doubling time assessment

Cells (35 000) were plated in 35 mm plates. The cell number was determined daily for 4 days. The doubling time was calculated from the numbers of cells between the first and the fourth day after plating.

Cell cycle analyses

Cells (both attached and floating) were harvested, counted, washed with PBS, fixed with 70% ethanol and stored up to 1 week at 4°C. The cells were then washed once with PBS, incubated in PBS containing RNase A (100 μg/ml, Sigma) for 1 h at 37°C, stained with propidium iodide (20 μg/ml, Sigma) and incubated on ice in the dark for 30 min. DNA content was analysed by Coulter Epics Altra Flow Cytometer (Beckman Coulter, Inc., Fullerton, CA). DNA cell cycle analysis software (MultiCycle, Phoenix Flow Systems, Inc., San Diego, CA) was used to quantify cell cycle distribution.

MSI analysis

293 Lα cells grown with 50, 0.2 and 0 ng/ml Dox were subcloned, and grown independently for 35 generations. The chromosomal DNA was extracted using the TRI Reagent (Molecular Research Center, Lucerne, Switzerland). MSI was assessed at the mononucleotide repeat locus BAT26. PCRs were carried out in a total volume of 25 μl containing ~100 ng of genomic DNA, as described by Loukola *et al*. (2001). The PCR products were diluted 1:4 and 0.5 μl was added to 10 μl deionized formamide (including 0.5 μl GS size standard 400 ROX), denatured at 95°C for 5 min, chilled on ice and loaded on a 96-capillary ABI PRISM 3700 DNA Analyzer (PE Applied Biosystems). MSI was defined as the occurrence of novel alleles that differed by ±3 nucleotides from the control (Loukola *et al*., 2001).

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Differential killing of mismatch repair-deficient and -proficient cells: towards the therapy of tumors with microsatellite instability.

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Advances in Brief

Differential Killing of Mismatch Repair-Deficient and -Proficient Cells: Towards the Therapy of Tumors with Microsatellite Instability

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Abstract

DNA mismatch repair (MMR) defects bring about a strong mutator phenotype and microsatellite instability (MSI). In an attempt to exploit MSI in cancer therapy, we constructed expression vectors carrying a thymidine kinase/blasticidin deaminase fusion gene downstream from a $(C)_{12}$ or an $(A)_{26}$ microsatellite and stably transfected these constructs into human cells in which the MMR status could be regulated by doxycycline. We now show that ganciclovir-resistant clones arising through frameshifts in the $(C)_{12}$ microsatellite were 20 times more frequent in cells in which MMR was inactivated. This difference may be exploited in gene therapy of tumors with MSI, which represent a substantial proportion of cancers of many different tissues.

Introduction

A substantial proportion of tumors of different organs displays MSI[1], a phenotypic trait characterized by a large increase in the frequency of frameshift mutations within repeated sequence elements, the so-called microsatellites. This anomaly is caused by inactivation of the postreplicative MMR system, which normally corrects strand misalignments arising in these repeats during DNA replication (1). In hereditary nonpolyposis colon cancer kindred, which represents ~5% of colon cancer patients, the MMR defect and MSI are linked to inherited mutations in genes encoding MMR proteins. In ~10% of sporadic colon cancers, MSI arises as a result of epigenetic silencing of the *MMR* gene *hMLH1* (2, 3), and an ever-increasing number of reports describe MSI also in cancers of head and neck, lung, prostate, breast, bladder, and other tissues (reviewed in Ref. 4). Past attempts to identify agents able to selectively kill MSI^+ cells largely failed. Upon treatment with a range of DNA damaging agents, substantial differences in the response of MMR-deficient and -proficient cells were observed only for cisplatin, which kills MMR-proficient cells ~3-fold more efficiently than MMR-deficient ones (5) and for S_N1-type methylating agents, where the difference is ~100-fold (6, 7). MMR-deficient cells were reported to be more sensitive to killing by CCNU than MMR-proficient controls (8), but this difference appears to be limited to only a subset of MMR-deficient cell lines. Thus, in an attempt to identify a more general approach toward the therapy of MMR-deficient tumors, we set out to exploit the MSI phenotype. In cultured human cells established from these tumors, MSI was reported to be two to three orders of magnitude higher than in control lines. We plan to introduce into the cells a toxin-encoding gene, the ORF of which is preceded by a labile microsatellite sequence such that the gene is out-of-frame (Fig. 1*A*). If the microsatellite undergoes insertion or deletion mutagenesis, the toxin ORF should in a given number of events be shifted into the correct reading frame; the construct should thus express the functional toxin polypeptide, and the transduced cell should be killed. This experimental strategy should result in an efficient elimination of MMR-deficient cells, whereas MMR proficient cells in which the microsatellite remains stable should be unaffected. However, before deploying the above strategy, we needed to establish an experimental system that would permit us to reliably test the relative stability of a variety of microsatellite repeats in MMR-proficient and -deficient cells. The critical characteristics of the ideal repeat should be its high stability in the former cells and substantial instability in the latter. The mutation frequencies of several microsatellites have been previously studied, using a variety of assays (9–12). However, in these studies, two major problems were encountered. First, it could not be excluded that the repeats acquired mutations already during the lengthy selection of the stable transfectants. Second, the studies did not use isogenic pairs of MMR-proficient and -deficient cell lines such that it was impossible to exclude the effects on the mutation frequency of other genetic defects present in these cells. Indeed, Hanford *et al.* (9) described extensive variation among microsatellite mutation rates of different clones of the same cell line. We now describe a system that successfully overcomes these drawbacks by making use of a strictly isogenic cell pair and a reporter system that allows for the elimination of mutated transfectants before the initiation of the experiment.

Materials and Methods

Cell Lines. 293T Lα cells were derived from the hMLH1-deficient human embryonic kidney 293T cells by stable transfection with a vector carrying the *hMLH1* cDNA under the control of the inducible Tet-Off expression system (7). The cells were grown in DMEM with Eagle salts (Life Technologies, Inc., Gaithersburg, MD), supplemented with 10% Tet System Approved Fetal Bovine Serum (Clontech, Palo Alto, CA), 2 mM L-glutamine (Life Technologies, Inc.), 100 IU/ml penicillin, 100 μg/ml streptomycin (Life Technologies, Inc.), 100 μg/ml Zeocin (Invitrogen, San Diego, CA), and 300 μg/ml hygromycin B (Roche Molecular Biochemicals, Basel, Switzerland). To obtain cells completely free of the MMR protein hMLH1 (293T Lα^-), the cells were transferred for at least 7 days to a medium containing 50 ng/ml DOX (Clontech). Fresh DOX was added every second day. To induce hMLH1 expression (293T Lα^+), the cells were transferred to a medium without DOX, the medium was changed the following day, and the cells were cultivated for at least 6 more days. Expression of hMLH1 in these cells fully restored MMR proficiency (7).

Vector Construction. The pSBCTKBSD vector (13) containing the fusion gene encoding thymidine kinase and blasticidin deaminase was used as a template for a PCR reaction in an assay consisting of 1× Cloned PFU buffer, 1 μM forward primer, 1 μM reverse primer, 200 ng of template DNA, 0.2 mM deoxynucleoside triphosphates and 2.5units/50μl reaction Pfu turbo DNA polymerase (Stratagene, La Jolla, CA). The primers (Microsynth, Balgach, Switzerland) were as follows: forward no-repeat, TGG CCA GGA TCC ACC ATG ATT GAA GAA TTC ATT GAA CAA GAT GGA TTG CAC GCA GG;

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[1] The abbreviations used are: MSI, microsatellite instability; BSD, blasticidin deaminase; DOX, doxycycline; GANC, ganciclovir; MMR, mismatch repair; ORF, open reading frame; TK, thymidine kinase; *TKBSD*, thymidine kinase/blasticidin deaminase fusion gene.

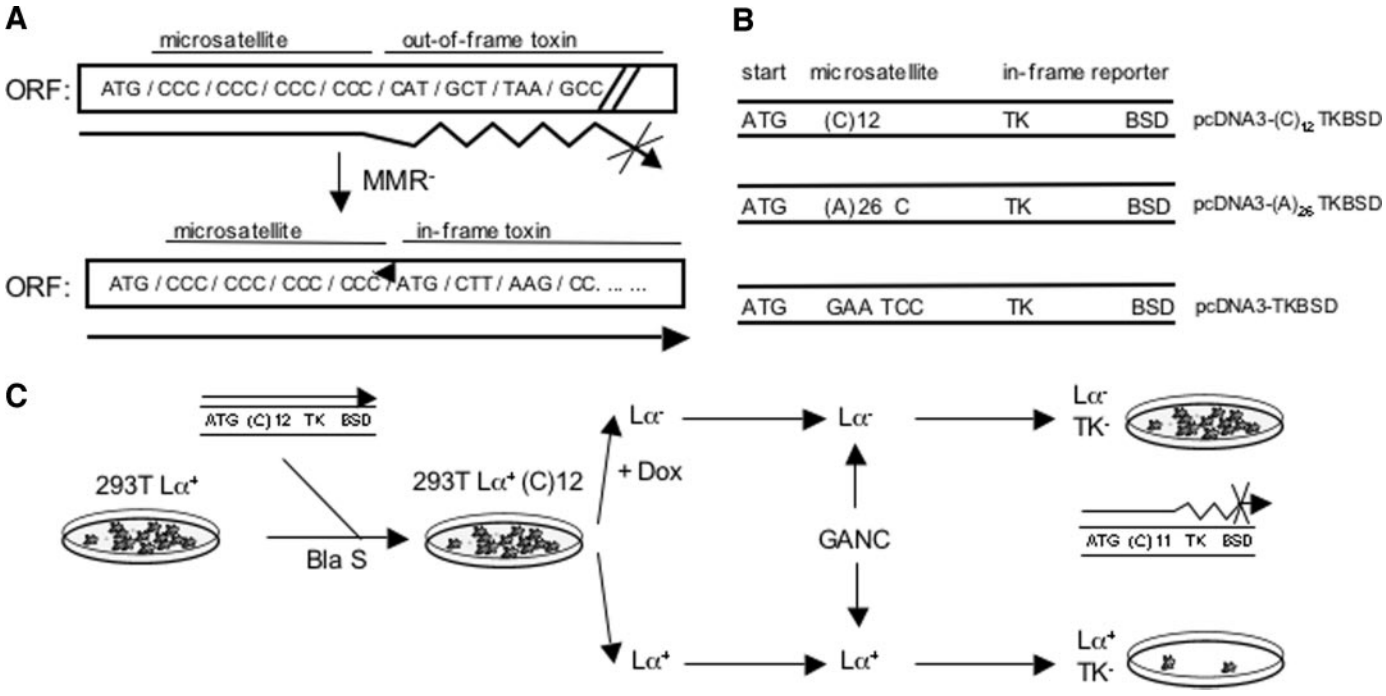

Fig. 1. *A*, scheme of a gene therapy approach designed to target MSI$^+$ cells. The microsatellite repeat puts the toxin out of the correct reading frame, and thus the vector produces no functional polypeptide. In a MMR$^-$ cell, the toxin gene may be reverted into its correct reading frame through MSI, and the cell will be killed. In contrast, the repeat should remain stable in cells with functional MMR. *B*, the constructs used in this study. The $(C)_{12}$ and $(A)_{26}$ microsatellites or a control DNA sequence without a repeat were inserted downstream from the start codon of the TKBSD fusion protein while keeping the ORF in its correct reading frame. *C*, scheme of the test assay (see also "Materials and Methods"). 293T Lα cells were transfected with constructs shown in *B*, and stable clones were isolated using blasticidin selection. The selected clones were then grown in two separate subcultures: in the MMR-proficient (Lα^+, without DOX) and -deficient (Lα^-, with DOX). After 4, 8, and 13 days (which corresponds to ~4, 8, and 13 cell generations, respectively), the cells were treated with GANC and plated to select for TK$^-$ mutants.

forward $(C)_{12}$, TGG CCA GGA TCC ACC ATG ATT GAA **CCC CCC CCC CCC** ATT GAA CAA GAT GGA TTG CAC GCA GG; forward $(A)_{26}$, TGG CCA GGA TCC ACC ATG ATT GTC **AAA AAA AAA AAA AAA AAA AAA AAA A**TT GAA CAA GAT GGA TTG CAC GCA GG; and reverse, TAC TCG CTC GAG TCA ATG TAT CTT ATC ATG TCT GGA TCG. The PCR cycle was as follows: 98°C for 3 min, (98°C for 1 min, 69°C for 1 min, and 72°C for 5 min)$_{30}$, 72°C for 10 min. The PCR products were digested with *Bam*HI and *Xho*I (both New England Biolabs, Beverly, MA) and cloned into the corresponding sites of pcDNA3 (Invitrogen), creating pcDNA3-TKBSD, pcDNA3-$(C)_{12}$TKBSD, and pcDNA3-$(A)_{26}$TKBSD vectors.

Isolation of Stable Transfectants. pcDNA3-TKBSD, pcDNA3-$(C)_{12}$TKBSD, and pcDNA3-$(A)_{26}$TKBSD vectors were digested with *Bgl*II and *Dra*III (both New England Biolabs) and subjected to preparative gel electrophoresis. The fragments containing the TKBSD fusion gene were isolated and used for transfection of 293T Lα cells using the FuGENE reagent (Roche, Basel, Switzerland). Selection was initiated 2 days after transfection with 10 μg/ml blasticidin S (Invitrogen). After 2–3 weeks, stable clones were isolated and additionally propagated with blasticidin (100 μg/ml).

Mutagenesis Assays. The selected clone, carrying the microsatellite repeat/*TKBSD* fusion stably integrated in the genome, was grown without or with 50 ng/ml DOX in a 6-well plate in a medium containing 100 μg/ml blasticidin for 7 days. At this time point, the cells grown in the presence of DOX were completely free of hMLH1 and thus MMR-deficient, and cells grown without DOX remained MMR proficient. The high concentration of blasticidin in the medium ensured elimination of cells with frameshifted inserts. The blasticidin was then removed and the cells were additionally propagated without or with DOX in a 6-well plate. As the doubling time is ~24 h, the cells were split every 2 days in a ratio 1:4 to maintain a constant cell number. In the absence of blasticidin, cells in which the *TKBSD* fusion gene was inactivated by frameshift mutagenesis (or otherwise) survived. Immediately upon blasticidin withdrawal and at the selected time points (4, 8, and 13 days, ~4, 8, and 13 generations), 1×10^5 or 5×10^4 cells were plated into 10-cm dishes in 10 ml of medium containing 30 μM GANC (Sigma, St. Louis, MO) to score for mutant (GANC-resistant) cells. At the same time, 300 cells were plated in a medium without GANC to assess plating efficiency (control). After 2 weeks of incubation, the colonies were stained with Giemsa (Fluka, Buchs, Switzerland) and counted (see Fig. 1*C* for a schematic outline of the experiment).

MSI Analysis. Chromosomal DNA from the GANC-resistant colonies was extracted using the TRI reagent (Molecular Research Center, Cincinnati, OH). The vector DNA sequence containing the repeat was amplified by PCR under the following conditions: 1× Taq buffer, 1 μM forward primer, 1 μM reverse primer, 300 ng of template DNA, 0.2 mM deoxynucleoside triphosphates, and 2 units/50 μl reaction TaqDNA polymerase (New England Biolabs). The following primers (Microsynth, Balgach, Switzerland) were used: forward (GCG GTA GGC GTG TAC GGT G), reverse (CCA GTC CTC CCG CCA CGA CC). The PCR procedure was as follows: 95°C for 2 min (95°C for 1 min, 60°C for 1 min, and 72°C for 1 min 20 s)$_{25}$, 72°C for 10 min. The PCR products were purified, and the DNA regions containing the repeats were sequenced using the primer GTA CGT AGA CGA TAT CGT CG on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Results and Discussion

The experimental system intended for use in gene therapy of tumors with MSI is based on transduction of the tumor cells with a vector carrying a toxin gene that is out-of-frame because of the insertion of a microsatellite immediately downstream from its AUG start codon (Fig. 1*A*). The inherent instability of the microsatellite in MMR$^-$ cells should result in restoration of the correct reading frame in a given percentage of the transduced cells and thus in expression of the toxin and cell death. However, for the purposes of the present study, we decided to invert this strategy by making use of an in-frame reporter/toxin combination that allows for a more accurate estimation of mutation frequencies and is free of artifacts (Fig. 1, *B* and *C*). The reporter gene construct was a fusion of *BSD* from *Aspergillus terreus* and *TK* from *Herpes simplex* virus (13). This fusion gene was preceded by a microsatellite repeat, which was inserted immediately downstream from the start codon, but which maintained the correct reading frame of the fusion gene. For the initial experiments, we chose

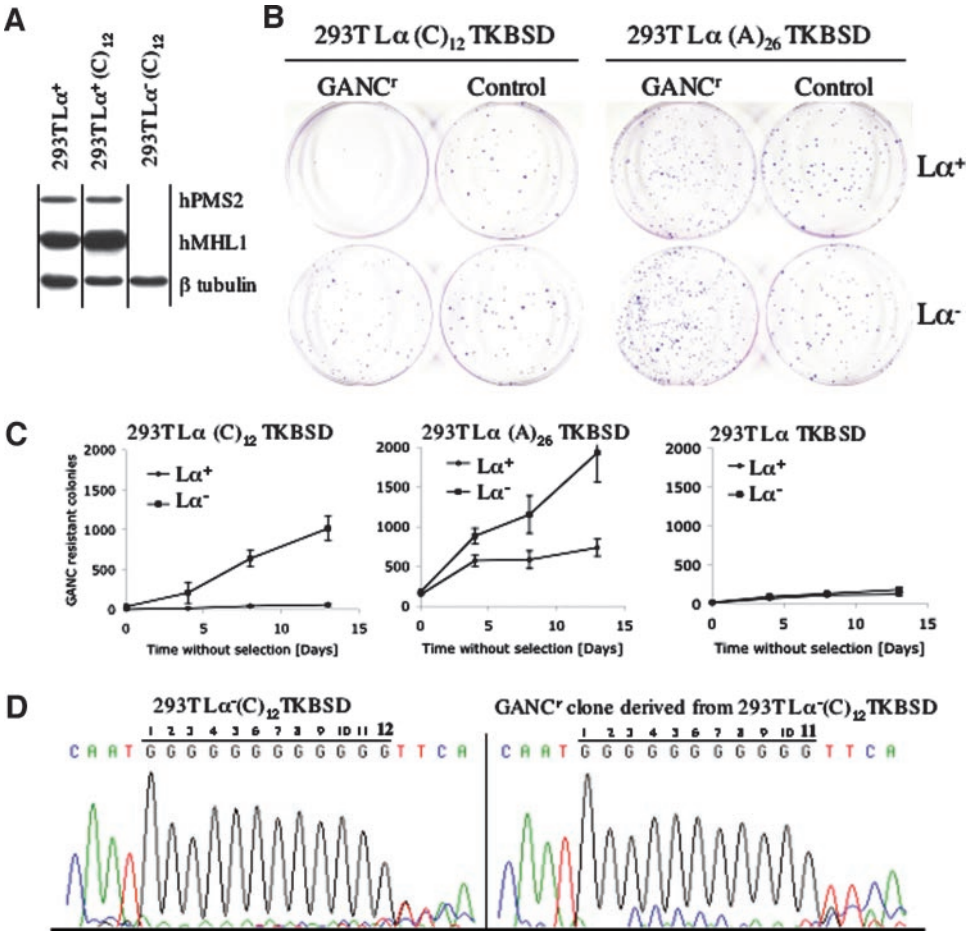

Fig. 2. Differences in repeat stability in isogenic 293T Lα cells. *A*, Western blot analysis of total cell extracts of 293T Lα $(C)_{12}$TKBSD cells cultured in the absence (Lα^+, expressing hMLH1/hPMS2) or presence (Lα^-, not expressing hMLH1/hPMS2) of 50 ng/ml DOX. The extracts of 293T Lα^+ cells are shown as the positive control. (See Ref. 7 for experimental details.) β-Tubulin was used to ascertain equal gel loading. *B* and *C*, comparison of repeat stabilities in isogenic MMR-proficient (Lα^+) and -deficient (Lα^-) cells. *B*, example of a typical result of an assay described in Fig. 1*C*. Cells were grown for 8 days without blasticidin, treated with GANC, plated, and the GANC-resistant colonies (GANC^r) were stained and counted after 2 weeks. Control, cells plated without GANC to estimate plating efficiency (see "Materials and Methods"). *C*, numbers of GANC-resistant colonies were adjusted, using plating efficiency, for 5×10^4 plated colony-forming cells, and plotted for each time point. Each data point represents the mean of five independent experiments; *error bars* show SD. D, MSI analysis. *Left panel*, original sequence without mutations; *right panel*, loss of one repeat unit.

the $(A)_{26}$ and $(C)_{12}$ repeats, together with a control construct that carried no repeat (Fig. 1*B*). The BSD protein confers resistance against blasticidin, which permits the selection of clones carrying the nonmutated construct stably integrated in the genome. Inducing MMR deficiency in one-half of the cells by adding DOX to the culture medium and propagating the cells independently in a MMR-deficient or -proficient mode for several generations without selection allows for mutations within the repeat to occur. Addition of GANC to the medium then eliminates cells in which no frameshifting occurred (Fig. 1*C*). Thus, by counting the surviving TK$^-$ (GANC-resistant) colonies, we can calculate the mutation frequency in a cell population that consisted initially exclusively of TK$^+$ cells (*i.e.*, without preexisting mutants), all carrying the vector integrated in the same genomic sequence context and in a strictly isogenic genetic background.

Before initiating this study, we had to check the integrity of the stable clones carrying the reporter constructs, more specifically, the integrity of the Tet-Off system that controls the inducible expression of hMLH1. As shown in Fig. 2*A*, the 293T Lα^+ cells and the clone stably transfected with the pcDNA3-$(C)_{12}$TKBSD vector [denoted 293T L$\alpha^+(C)_{12}$TKBSD] expressed both hPMS2 and hMLH1 in similar amounts. In the presence of DOX, the transcription of hMLH1 was shut off, which resulted in the depletion of the hMLH1/hPMS2 heterodimer (7); these cells are denoted 293T L$\alpha^-(C)_{12}$TKBSD. The selected clones carrying the pcDNA3-$(A)_{26}$TKBSD and pcDNA3-TKBSD vectors, 293T L$\alpha(A)_{26}$TKBSD and 293T Lα TKBSD, respectively, behaved similarly (data not shown).

Results of the mutagenesis assays ("Materials and Methods") indicated that the $(C)_{12}$ repeat remained stable in the MMR-proficient 293T Lα^+ cells (Table 1 and Fig. 2, *B* and *C*). The *TK* gene remained in-frame, and upon addition of GANC, most of the cells were killed. In contrast, frequent frameshift mutations within this repeat in a MMR-deficient background gave rise to a ~20-fold higher number of GANC-resistant clones in which the *TKBSD* gene was shifted out-of-frame (Fig. 2, *B* and *C*). The number of GANC-resistant colonies increased with time in both MMR-proficient and -deficient backgrounds, but the fold-difference remained relatively stable (Fig. 2*C*). In contrast to $(C)_{12}$, the $(A)_{26}$ repeat was labile in both MMR-proficient and -deficient backgrounds, displaying only ~2 fold difference in stability (Fig. 2, *B* and *C*, and Table 1).

To confirm that the GANC-resistant phenotype resulted from a mutation in the reporter construct, we sequenced the DNA regions containing the repeat and its close proximity. Microsatellite frameshifts were detected in all of the 293T Lα^- samples sequenced (30 of 30). Only -1 frameshifts were observed in the $(C)_{12}$ repeat and -1 or, less frequently, -2 frameshifts in the $(A)_{26}$ repeat (Fig. 2*D*).

Table 1 *Average fold-differences in the number of GANC-resistant colonies in MMR-deficient versus proficient background*[a]

Days without selection	293T Lα		
	$(C)_{12}$ TKBSD	$(A)_{26}$ TKBSD	TKBSD
4	29.5 ($\pm$17)	1.6 ($\pm$0.1)	1.4 ($\pm$0.1)
8	17.1 ($\pm$3.9)	2.1 ($\pm$0.3)	1.2 ($\pm$0.1)
13	19.2 ($\pm$2.8)	2.6 ($\pm$0.2)	1.6 ($\pm$0.1)

[a] The values shown in the table were calculated by dividing the number of GANC-resistant colonies in the MMR-deficient background by the number of GANC-resistant colonies in the MMR-proficient background at each time point (4, 8, and 13 days) for each individual experiment. Shown are average values for each time point from five independent experiments. See also "Materials and Methods." Numbers in parentheses show SEs.

Table 2 *Average mutation frequencies (fractions of mutated cells/generation)*[a]

293T Lα	MMR$^+$	MMR$^-$
$(C)_{12}$ TKBSD	$8.0\ (\pm 1.0) \times 10^{-5}$	$1.6\ (\pm 0.1) \times 10^{-3}$
$(A)_{26}$ TKBSD	$8.2\ (\pm 0.6) \times 10^{-4}$	$2.6\ (\pm 0.3) \times 10^{-3}$
TKBSD	$1.7\ (\pm 0.1) \times 10^{-4}$	$2.4\ (\pm 0.2) \times 10^{-4}$

[a] The values were obtained by plotting the number of GANC-resistant colonies against time for each individual experiment. The increment of GANC-resistant colonies/doubling time (22 h) was calculated from the regression curve and was divided by the number of cells plated (corrected for plating efficiency). The results are based on five independent experiments. See also "Materials and Methods." Numbers in parentheses show SEs.

Although most of the DNA samples isolated from the GANC-resistant MMR-proficient cells also contained -1 or -2 microsatellite frameshifts, other types of mutations (3 of 30) were also detected (data not shown) in agreement with previous studies (9). In control clones not containing a repeat within the reporter gene, the construct remained relatively stable and we did not detect significant differences in stability between MMR-proficient and -deficient backgrounds (Fig. 2*C* and Table 1). The above information is invaluable for the design of the therapeutic out-of-frame vector (Fig. 1*A*). In theory, only a fraction of frameshift mutations should lead to the restoration of the correct reading frame because of the possibility of both insertions and deletions. Our data demonstrate that deletions of a single repeat unit of a given microsatellite repeat predominate. Taking this evidence into account, it should be possible to design vectors with a high propensity toward shifting into the correct reading frame, *i.e.*, by having the toxin gene insert in the vector in a $+1$ reading frame in cases where it is preceded by a mononucleotide repeat.

Microsatellite mutation frequencies measured in our MMR-deficient cells (Fig. 2*C* and Table 2) roughly corresponded to those described by others (9, 14, 15). However, the relative differences between MMR-proficient and -deficient cells were somewhat smaller: ~20-fold in our system as compared with 16–340-fold as described by Hanford *et al.* (9) or 25–100-fold as described by Kahn *et al.* (12). However, the latter studies compared mutation frequencies of MMR-deficient colon carcinoma cells either with those of unrelated MMR-proficient colon carcinoma cells or even with those of MMR-proficient cancer or normal cells of different type. It has been well documented that cells acquire a plethora of mutations during transformation. Some of these mutations might inactivate cell cycle checkpoint pathways, which might allow DNA replication in the presence of DNA damage, and thus permit the accumulation of additional mutations even in a MMR-proficient background. Indeed, Boyer and Farber (16) found a 75-fold difference in the mutation frequency of the same microsatellite in MMR-proficient normal human fibroblasts and fibrosarcoma cells. Clearly, genetic differences between cells of different origin make a direct comparison of mutation frequencies very difficult.

A recent study (10) used the human MMR-deficient colon cancer cells HCT116 and their MMR-proficient counterparts (HCT116+Chr3) where the MMR defect was corrected by chromosome 3 transfer. These two cell lines, although not isogenic, are more closely related than those used in the studies cited above. Interestingly, the observed 30-fold difference in the stability of a $(CA)_{13}$ microsatellite is quite close to that of the $(C)_{12}$ repeat examined in our study.

The 20-fold difference in the stability of the $(C)_{12}$ microsatellite between MMR-proficient and -deficient cells is lower than might have been anticipated from the results of earlier studies, however, given the extremely low mutation frequency in the MMR-proficient cells, it is likely to be therapeutically exploitable. Moreover, it is highly likely that analysis of a larger number of mononucleotide and dinucleotide repeats will identify a sequence with a substantially higher therapeutic index. Thus, exploitation of the MSI phenotype, which is currently estimated to segregate with ~15% of colon cancers, may represent a valid approach toward combating these tumors and, more importantly, their metastases.

One of the major challenges of tumor therapy is acquired resistance to treatment. Because the MSI phenotype is linked with defective MMR, the only chance the transduced cell has to escape death is either to stop replicating, which would in itself lead to tumor regression, or to silence the transcription of the transgene. The latter scenario is unlikely because gene silencing requires as a rule many cell divisions and because the microsatellite repeat tested in our study was unstable after only four replication cycles. Even if this problem should arise, it could be overcome by repeated transductions. It is therefore likely that the problem of resistance will not pose a substantial threat to this approach.

The study described above represents but an initial step toward this goal. As the environment of cells in tumors differs dramatically from that in cell culture, it will be necessary to carry out *in vivo* experiments using, in the first instance, human tumor xenografts in nude mice. Should these experiments meet with success, the transducing vector will be remodeled to carry the suicide gene out-of-frame, which would be moved into the correct reading frame through selective frameshift mutagenesis in MSI cells, as shown in Fig. 1*A*. In a gene therapy setting, most cells in solid tumors are not transduced and it is likely that not each cell will mutate the microsatellite. But as tumor cells are in close contact, it is anticipated that the suicide gene will exert a bystander effect, which should bring about the death not only of the transduced cell that acquired the frameshift mutation but also of a number of surrounding cells (17). A complete eradication of a tumor expressing TK has been reported, although only 10% of the tumor cells expressed the enzyme (17). Cytosine deaminase, another suicide gene frequently used in gene therapy trials, has been reported to be effective even if only 2% of the tumor cells were transduced (18), and strategies causing even more effective bystander effects are being developed (19). The system described in this study should permit the identification of the most effective microsatellite/enzyme/prodrug combination that could then be further developed for therapeutic use.

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5.Conclusions and future perspectives

5.1. The role of hMLH3 in MMR

Our data represent the first report on the variable expression levels of hMLH3 in human cell lines and its possible epigenetic inactivation by promoter hypermethylation. We also confirmed, by immunoprecipitation experiments, the *in vivo* interaction between hMLH1 and hMLH3. An interesting observation was the different stability of hMLH3 in Sf9 and in human cells. While the protein needs the presence of hMLH1 when expressed in insect cells, it is instead stable in hMLH1-deficient cells, suggesting the requirement of additional interacting partners *in vivo*, to mediate its stabilization. We thus plan to use the Tandem Affinity Purification method to identify proteins in complex with hMLH3 and so to further characterize the role of hMLH3 or hMutLγ in human cells. Most importantly, we could demonstrate an *in vitro* activity of hMLH3 in MMR. The contribution of hMLH3 to MMR seems to be marginal thus the significance of this contribution *in vivo* is questionable. Interestingly, parallel to our observation, Lipkin et al. demonstrated the contribution of Mlh3 to tumour suppression in the mouse. In summary, more data is needed to clarify the role of hMutLγ *in vivo*, especially regarding its role in colon cancer.

5.2 Identification of interacting partners of hMLH1 and hPMS2 by Tandem Affinity Purification

We used the Tandem Affinity Purification (TAP) technique to identify novel interacting partners of hMLH1 and hPMS2. Our analysis resulted in the identification of several known interacting partners of hMLH1 and/or hPMS2, thus validating the method, and revealed the presence of numerous, previously undescribed, proteins in complex with one or both MutL proteins. We grouped these proteins into functional groups and we set out to characterize some of these interactions further. Remarkably, we identified the BRCA1-

interacting protein 1 (BRIP1 or BACH1), recently described as FancJ, in complex with hMutLα. This interaction was validated by reciprocal immunoprecipitation and it is of particular interest. BRIP1 was shown to have DNA helicase activity and it has been implicated in the repair of double strand breaks. Notably, the helicase involved in human MMR, if any, still remains to be identified and we are currently investigating the possible role of BRIP1 in MMR or related-processes.

In addition, we validated by immunoprecipitation analysis the interaction between hMLH1 and Angiomotin. Angiomotin, a recently identified protein involved in the promotion of angiogenesis, is present in the cells in two forms, a high molecular weight (p130) and a lower molecular weight (p80). Interestingly, only the p130 form seems to bind to hMLH1. Immunofluorescence experiments showed Angiomotin to be localized in the cytoplasm of 293T cells, we thus assume that hMLH1 might assist Angiomotin in its function rather then the contrary. We intend to start collaboration with Prof. Lars Holmgren, (Karolinska University Hospital, Stockholm) to investigate this unexpected cytoplasmic role of hMLH1.

The finding of Importin α2 in complex with hMLH1 and hPMS2 points to a role of this protein in the nuclear import of hMutLα. Importin α2 belongs to the family of karyopherins, a group of proteins devoted to the nuclear import of polypeptides containing a sequence called nuclear localization signal (NLS). Both hMLH1 and hPMS2 were shown to possess a NLS in their sequence so that the possibility that Importin α2 is the molecule involved in their nuclear translocation is attractive and will probably be one of our future subjects of investigation.

5.3 Identification of hPMS1 interacting partners by large-scale immunoprecipitation

The analysis of the interacting partners of hPMS1 brought us to the identification of several proteins. Among these the presence of several factors belonging to the ubiquitin-pathway is of particular interest, suggesting that hPMS1 might undergo such a post-translational modification. We investigated this possibility and demonstrated that hPMS1 is in fact poly-ubiquitinated in untreated cells. This could be a relevant finding for a possible role of hPMS1 in human cells. Interestingly, recombinant hPMS1 lacked any MMR activity *in vitro*. We are now investigating whether the activity of hPMS1 in the repair process is dependent on its ubiquination.

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7. Curriculum Vitae

Personals

Name	Elda Cannavo
Born	22 December 1972, Catania, Italy
Nationality	Italian

Education

1986-1991	Gymnasium: Liceo Scientifico "P.Ruffini", Viterbo, Italy
1991	Final exam "matura", distinction 60/60
1991-1997	Studies at University of Pisa, Italy, Faculty of Biological Science Specialization in Molecular Biology and Virology
1997	Final diploma *cum laude* in Biological Sciences, University of Pisa, Italy. Defence of the thesis. Title of diploma thesis: "Human herpesviruses 6 and 7 (HHV-6 and HHV-7) in healthy donors and in HIV-1 infected patients: molecular quantification of HHV-7 viral load"
1999	Additional state exam in Biological Sciences, University of Viterbo, Italy
2000	Advanced 6 months course in Immunology at the Hospital "San Carlo di Nancy", Rome, Italy

Professional Training

1996-1997	Preparation of diploma thesis at the Department of Biomedicine, Virology section, University of Pisa, Italy
1997-1999	Practising at the Department of Neurosciences, laboratory of Pharmacology, "Tor Vergata" University of Rome, Italy
1999-2001	Research Fellow, laboratory of Pharmacology, "Istituto Dermopatico dell'Immacolata", Rome, Italy
2001-2002	Visiting scientist at the Institute of Molecular Cancer Research, University of Zurich, Switzerland
2002-2006	PhD student at the Institute of Molecular Cancer Research, University of Zurich, under the supervision of Prof. Dr. Josef Jiricny

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